nature

More worries about the pill

Two new studies have provided discouraging suggestions that oral contraceptives may entail previously unrecognized (but small) risks to women's health. Panic is not called for, straight talking is.

FOR the best part of two decades, the pill has been almost the perfect contraceptive. Although originally intended as materials that might help to contain the growth of population in developing countries, the various cocktails of synthetic steroid hormones now marketed as oral contraceptives for women have probably been more influential among the health-conscious women of prosperous industrial societies. And most of the consequences have been benefits. For a large proportion of women in advanced societies, the planning of a next pregnancy has become a conveniently attainable goal, with all the social and personal civility that follows. Nobody can know how great a contribution the pill has made to the relative stability of the populations of advanced societies, but certainly it is a long time since prosperous governments have been alarmed at the rate of growth of population from reproduction (as distinct from longevity). This does not imply that the pill has been blameless. Some have argued that freedom from the tyranny of pregnancy by accident is responsible for recent changes in sexual behaviour among young people, probably putting the cart before the horse in the process. More to the point, in the past few years it has been recognized that the prolonged use of oral contraceptives may entail the risk of circulatory problems in older women. Now, in Britain, there is a wave of excitement over two reports suggesting that use of some kinds of oral contraceptives by much younger women may increase the risk of cancer of the breast and of the cervix. In the spirit of the dictum that there is no such thing as a free lunch, people are saying that the pill was almost too perfect to be true.

The obvious danger now is that people, especially younger women, may panic. In reality, the studies now reported are nevertheless (and through no fault of their authors) incomplete and, for the time being, inconclusive. The reason is simply that too little time has passed since oral contraceptives have been widely used for the results of prospective epidemiological surveys to be decisive. Only several years from now will it be possible for physicians to give their patients a rounded assessment of the risks of using oral contraceptives. But already a modest change seems called for in what patients should be told. Hitherto, a physician's response to the question "Will the pill increase the risk of my contracting some kind of cancer?" should properly have been "I don't know — there is no evidence one way or the other". Now the proper response is a little less encouraging, something like "There's some evidence that it will, but there is no way yet of telling how great the extra risk may be but it's probably quite small".

Breast cancer

This is a simple reading of the report by Professor M. C. Pike and a group of colleagues at the University of Southern California School of Medicine at Los Angeles, published last week in *The Lancet* (ii, 926; 1983). The study they have carried out is technically a case-control study in which the cases of breast cancer occurring among younger women (younger than 33 years or, in the latter phases of the study, 37) were matched with apparently normal women of the same age living in the same neighbourhood. The objective of this study was to find out whether the histories of the two groups of women differed from each other in ways that might significantly account for the difference of cancer incidence between them. The conclusion is that the two groups do indeed differ significantly in their past use of oral contraceptives. In particular, the group of women in whom breast cancer had developed differed from the control group in their greater past use of oral contraceptives, whence the inference that oral contraceptives increase the risk that breast cancer will develop at a relatively early age.

As always on these occasions, the simple statement is less than half the truth. First, as Pike and his colleagues say, other similarly constructed studies have led to the conclusion that there is no correlation between the use of oral contraceptives and the later occurrence of breast cancer. In such circumstances, it is however meaningless to ask which of the studies is the better guide to action. There is no reason to suppose that any of them is technically unsound. The differences between them may in the end be explained by systematic differences between the populations from which the groups of women were drawn but may also be strictly statistical, for successive measurements of a statistically defined variable do not always yield the same result.

High progestogen

What makes the new study persuasive, however, is that there is a positive correlation between the use of oral contraceptives and the later development of breast cancer only for those combination contraceptives based on both oestrogen and progestogen in which the second ingredient is relatively potent (as measured by a biological assay). The point is important because there is a possible explanation of the observed phenomena as well as of the earlier observation that exactly the same materials actually protect their users against the risk of ovarian cancer and of cancer of the endometrium of the ovary. A second reason for taking the results of Pike *et al.* seriously is that there appears to be a positive correlation between the duration of contraceptive use and the risk of subsequent breast cancer as well as between first use of these oral contraceptives before the age of 25. Their own conclusion is that high-progestogen pills before the age of 25 can substantially increase the risk of subsequent breast cancer, and that the risk increases with the length of time for which these oral contraceptives are used.

So how great is the extra risk? This is what patients will be asking of their physicians. And the perplexing answer is that while this general account of the findings of the survey may be qualitatively correct, very little in the way of statistical significance can be attached to estimates of relative risk based on such a small sample. Among the 314 women with breast cancer, 24 were found to have taken high-progestogen contraceptives for more than six years, while only seven of the control group had used similar materials for such a length of time. On the face of things, that suggested a five-fold increase of the risk of breast cancer among the women taking contraceptives — but nobody would be surprised if the true figure were less than two or more than ten. In other words, what the data show is that there is a need for the recruitment of more women suffering from breast cancer to studies of this kind, for their extension to later ages and for independent approaches to the problem of estimating risk.

The parallel study now reported (*Lancet* ii, 930; 1983) by Professor M. P. Vessey and a group of colleagues from the University of Oxford is more easily interpreted in that it consists of a comparison of the incidence of cervical cancer in more than 6,000 women using pills as contraceptives and another 3,000 or so

0028-0836/83/430749-02$01.00

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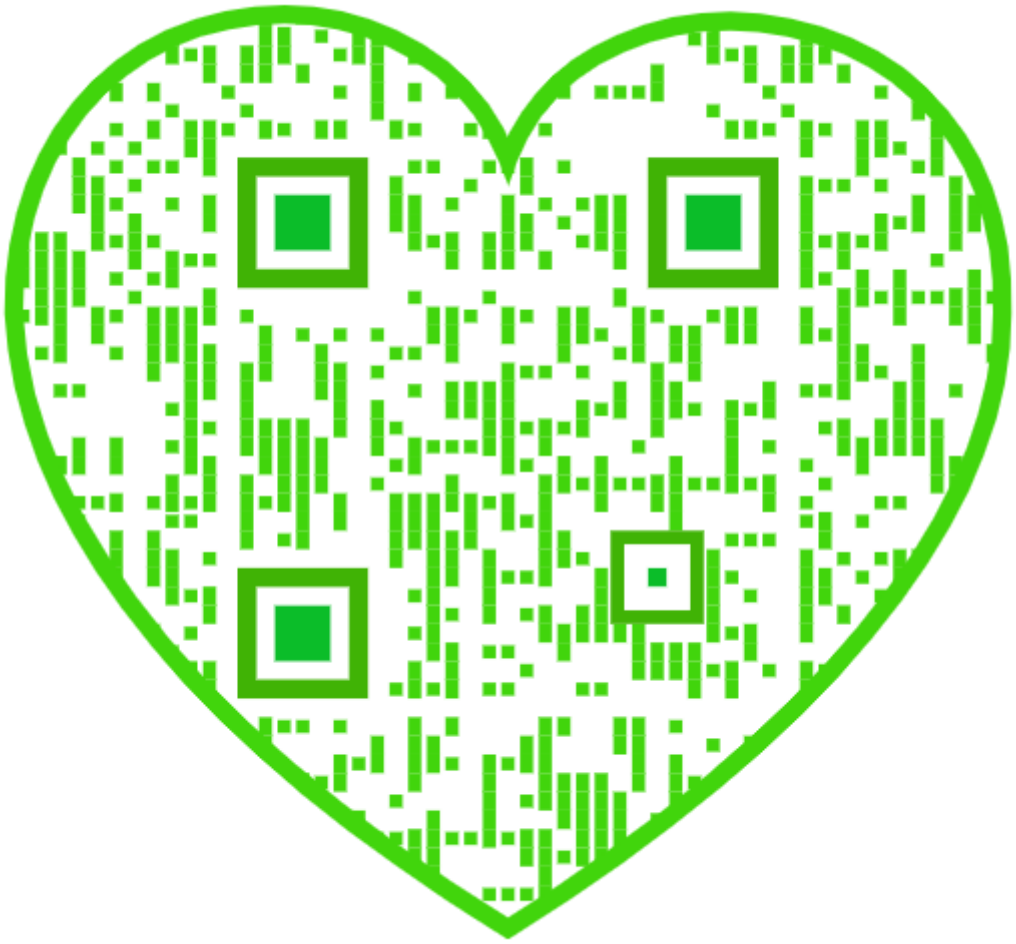

relying on intrauterine devices (IUD). This study has the advantage of providing its own built-in control group, but the age-distribution covers a wider range than in the Los Angeles study, so that the results must necessarily be less specific. The outcome is, however, again discouraging — all 13 cases of cervical cancer were found among the group using oral contraceptives. Again there seems to be a correlation between risk and duration of use but nobody would go to the stake in defence of any particular estimate of the risk. And again, the absolute risk is small — perhaps a doubling of the natural rate of something like 0.1 per cent.

The lessons to be drawn from this important work are several. First, for individual women wondering how with least risk to safeguard against unwanted pregnancy, especially when they are young, there is no substitute for a realistic appraisal of the small risks entailed and for an intelligent appraisal of the benefits and snags of other methods of contraception. As with other effective drugs, it would be surprising if oral contraceptives were free from side effects. But the risks are absolutely small — much smaller than those of regular cigarette smoking, for example. A decision to use oral contraceptives is neither irrational nor suicidal. For physicians, the new developments are yet another proof of how complicated the modern world has become — yet there is no civilized alternative to an honest attempt to explain what are the new developments, and to suggest what may lie ahead. Only public health authorities have a clear course before them — better facilities for the early diagnosis of the tractable forms of cancer, not simply those of the breast and cervix, are an urgent need. Will they be provided as generously as the circumstances require? □

Greenhouse research

There is a need for more research on CO_2 and climate. It should be directed where it matters.

Is the climate about to change because of the accumulation of carbon dioxide in the atmosphere? It is perhaps inevitable that the Environmental Protection Agency and the National Academy of Sciences in the United States should differ in their opinions on the future as sharply as reported on the next page. Much the same happened some years ago, when people were worried about the consequences for the ozone layer of the release of chlorofluorohydrocarbons into the atmosphere. But surely, there must come a time when further study is inappropriate, and when something will have to be done? Indeed, but not yet.

The academy report is itself a splendid pointer to the further study now needed, partly because of its omissions. First, there is general agreement on the fact of the accumulation of carbon dioxide — measurements are now much improved, and differences between predictions of future accumulations, based on different economic models dictating fossil fuel use, are not small but affect only the time-scale of events. There is more bite in what the academy's report has to say on interactions between the atmosphere and the oceans — solution of carbon dioxide accounts for 40 per cent of carbon dioxide released into the atmosphere by processes that are now reasonably well understood, and which are likely to become less efficient as concentrations in the atmosphere and surface layers increase. The potential role of the oceans as a massive heat sink for cooling the atmosphere is, by comparison, only poorly understood and cries out for further study.

On the evidence of this latest study, however, the greatest uncertainties attend the attempt to calculate what will be the climatic consequences of future carbon dioxide accumulation. On the grounds that a comprehensive survey of this part of the problem was published only in 1982 under the same auspices, this year's academy committee merely quotes the earlier study to the effect that the prospective warming of the surface of the Earth by the greenhouse effect is unlikely to be substantially reduced by negative feedback (such as could be caused by clouds). This is merely another proof that among the programmes of study now called for, the construction of more realistic models of the atmosphere is the most urgent — if the most difficult. □

Where was Hoyle?

It is not in bad taste to suggest another 1983 Nobel prize, sadly overlooked.

LAST week's clutch of Nobel prizes is more than usually interesting, as was that two weeks ago to Dr Barbara McClintock. In each case, the nominating committees and the Royal Swedish Academy seem to have gone out of their way to find people who are not only distinguished for the importance of their scientific work but who are also in some way acknowledged to be remarkable for the durability of their contribution to the understanding of science. The saga of Dr McClintock's long battle against prejudice and incomprehension is an heroic tale. Taube (this year's chemist) is plainly one of the most versatile of people. Chandrasekhar (one of this year's two physicists) has also in his time surmounted prejudice, mostly at Cambridge in the 1930s (and what a collaboration there would have been if Eddington had recognized that his younger colleague had such tools in his gift). But William Fowler (the other physicist) belongs in this group, even though his sunny temperament and engaging personality can never have invited prejudice, because of the distinction of his physics — especially his early decision in 1946 to embark on a study of nuclear reactions likely to be important on the Sun — as well as for his lasting influence on a generation of physicists at the California Institute of Technology. It may be especially important for the future that the Nobel committees have finally agreed that astrophysics is now properly within the scope of physics.

For all those reasons, it is important that what follows should be read with care, as meaning literally no more than what it says — and especially by those whose names are mentioned, all of whom are great friends of this journal. To adapt a now well-known phrase, however, it cannot have escaped most people's notice that the list of physics prizes does not include some of those whose names naturally come to mind in connection with the problem of nucleogenesis, several of whom are mentioned on page 759 and the most obvious of whom is Sir Fred Hoyle. To say this is in no sense to suggest that Fowler's work is unimportant or undeserving — on the contrary, everybody will be delighted that his massive contribution to physics has been acknowledged in this way. Fowler, generous man that he is, would probably have been even happier if he and his old collaborator had been joint prizewinners.

So what went wrong? It is entirely possible that the Nobel committees, inhibited by their by-laws, are saving up the names of contributors to the nucleogenesis problem for some future years, but given the pace of discovery, that would be a risky manoeuvre. It is also possible that the committees, or even the Royal Swedish Academy, were impressed that Hoyle differs from Fowler (and most other prizewinners) in that, always subversive as the best scientists are, he has recently been over-subversive, advocating with too much passion and too little evidence the unnecessary theory that living things on the surface of the Earth originally arrived from intragalactic space. By the general consent of this journal's referees, that is where the weight of opinion lies. Yet it would be a shabby circumstance if such considerations or fears of the embarrassment there would be if an unrefereed account of this theory were blazoned across the world at some prizegiving ceremony had deflected the committees from the logic of what might have been their intentions.

It would be improper but also impracticable to demand that the committees should at some point in the future make amends. If the Nobel prizes are to retain their high reputation, each year's prize must go to that year's obvious candidates. And nothing in the preceding sentences should be taken to mean more than the obvious — that Hoyle would have been a natural partner for Fowler, that the contributions of the other partners to their famous collaboration are in no sense belittled and that especially in the supposedly adventurous discipline of physics, it is unreasonable to expect that able men and women will always be people whose genius is never marred by eccentricity. □

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Atmospheric carbon dioxide

Two views on whether more means doom

Washington

TWO new studies of the "greenhouse effect" of atmospheric carbon dioxide raised the political temperature in Washington last week but added no surprising new information. A report by the Environmental Protection Agency (EPA), predicting a 2°C rise in global temperature by about the year 2040, was denounced by President Reagan's science adviser, George Keyworth, as "unnecessarily alarmist". But Keyworth praised a report by the National Academy of Sciences which said that a doubling of atmospheric carbon dioxide levels by late in the next century would cause a temperature increase of between 1.5 and 4.5°C.

Keyworth's reaction to the EPA study appears to be based as much on the tone of the report as on its conclusions. Using existing models of the world economy and energy use, the report says that as a "best guess", atmospheric carbon dioxide concentrations will reach 590 p.p.m. by 2060, roughly double pre-industrial concentrations. There would be a 2°C temperature increase by around 2040 and the total increase of temperature would approach 5°C by 2100. But the report stresses that the projections are extremely uncertain and that the rate of increase of other greenhouses gases, such as methane and chlorofluorocarbons, could greatly influence the timing of a temperature increase.

The National Academy report, also warning of large uncertainties in its projections, concludes that the concentration of atmospheric carbon dioxide will most probably double to more than 600 p.p.m. in the third quarter of the next century, with a 1 in 20 chance that the doubling will occur before 2035. The impact of such a doubling on the climate is hard to assess because of "fundamental gaps" in knowledge about the interaction between the atmosphere and the oceans, but the report suggests a warming near the lower half of the range of between 1.5 and 4.5°C.

The disparity between the EPA projections and those of the academy is not large and can be explained in part by the academy's use of a sophisticated new model of the energy economy that produces a lower rate of carbon dioxide emissions than most earlier studies. The model suggests that carbon dioxide emissions will grow by about 1.6 per cent annually until 2025 and that growth will then decrease to about 1 per cent. What appears to have irritated Keyworth is not EPA's projections themselves but its warning that early planning is necessary to deal with the disruptive effects of a global rise in temperature.

The National Academy report argues against immediate changes in energy policy and emphasizes the need for more research. While expressing "deep concern" about the possible range of environmental changes, the academy says the need is for caution rather than panic, and warns that hasty policies based on inadequate research could be costly and ineffective. It suggests that research and development should give "some priority" to future energy options not based on burning fossil fuels, but says there is no immediate reason to change the existing mix of fuels.

In contrast, EPA describes the potential changes in climate in the next century as potentially "catastrophic" and says that expeditious planning, as well as continued research, is essential. These recommendations, rather than the projections on which they are based, were singled out for criticism in a statement issued by the White House Office of Science and Technology Policy (OSTP). The federal government is well aware of the greenhouse problems, and is currently spending $20 million on research into the relationship between carbon dioxide and the climate, OSTP said. EPA's suggestion that planning is urgently necessary was "unwarranted".

The severity of OSTP's reprimand is puzzling. The "planning" called for by EPA does not appear to mean anything more dramatic than research on how to adapt to environmental changes, and the report explicitly rules out immediate changes in energy policy. Most of these, such as worldwide taxes on fossil fuels or a ban on synfuels and shale oil, would have little or no effect on the rate of warming, the report says. Only a total ban on coal would have a significant effect, but that would not be politically and economically feasible.

Also ruled out in the EPA report are the prohibition of carbon dioxide emissions (too expensive); afforestation (would cause too much competition for land, fertilizer and irrigation); and injecting sulphur dioxide into the stratosphere to reflect solar energy (still too many unknown side effects). Dr Keyworth's angry reaction may turn out to have less to do with the substance of what EPA said than with the fact that it appears to have said it without clearing its views first with the rest of the administration.

Peter David

AIDS

Plan for European centre agreed

A MEETING of 130 government representatives organized by the European regional office of the World Health Organization agreed last week to set up a European collaborative centre on acquired immune deficiency syndrome (AIDS). At the meeting, held at Aarhus (Denmark), Denmark, France and West Germany offered to accommodate the proposed centre, but a final decision will be made only when other governments have had a chance to respond to the proposal.

The objective of the centre is to create a European equivalent of the service rendered in the United States by the Centers for Disease Contol at Atlanta, Georgia, which have been influential in defining criteria for the diagnosis of AIDS, for providing advice to physicians and the general public and for keeping track of the spread of the disease.

The number of AIDS patients in Europe was estimated at last week's meeting to be 267, roughly ten per cent of the number of cases in the United States. While the pattern of the disease (primarily occurring among male homosexuals, drug addicts and recipients of blood transfusions and blood products) is similar in Europe and the United States, European physicians are disturbed by what was called last week "the African connection", thought to account for the relatively high incidence of the disease in France (94 cases) and Belgium (38 cases).

The conference last week agreed to accept as a basis for the identification of AIDS patients the diagnostic criteria promulgated at Atlanta last year. For the rest, the conference accepted that there has been no progress in understanding the aetiology of AIDS nor in the treatment of the condition.

Meanwhile, one graphic illustration of the pitfalls of over-eager interpretation of data bearing on the causation of AIDS was provided last week in *The Lancet* (**ii**, 963; 1983) by a group of pathologists from the Queen's University of Belfast, commenting on a report earlier this year of virus-like particles in electron micrographs of cells from affected patients (Feremans, W. *et al. Lancet* **ii**, 53; 1983) and a later account (*Nature* **305**, 264; 1983) of the excited response of Belgian newspapers to that information. Dr Tom Gardiner and two colleagues from Belfast now point out that the supposed virus-like particles are virtually identical with organelles known as multivesicular bodies or, alternatively, as endosomes, and whose function appears to be to internalize NAD to reprocess complexes of external receptors with the ligands attached to them (see *Nature* **305**, 684; 1983). Gardiner and his colleagues point out that endosomes (or receptosomes) are found in cells from all kinds of tissues, lymphocytes included. □

0028-0836/83/430751-01$01.00

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Star wars

Reagan gets green light

Washington

BARRING a mutiny in Congress — and nothing is impossible during a presidential election year — the United States seems ready to pay next year the first instalment of a multibillion dollar programme to develop a system of space-based defences against ballistic missiles. A senior interagency group has told President Reagan that his vision of a futuristic shield against nuclear attack, outlined in his "star wars" speech last March, could be made to work.

The group's report, leaked last week in *Aviation Week and Space Technology,* recommends pressing quickly ahead with research into new technologies so that the feasibility of the idea can be demonstrated by the early 1990s. The aim would be to destroy Soviet missiles during their boost phase immediately after launch or, failing that, while they were mid-course or re-entering the atmosphere.

Interception during the boost phase, before the attacking missiles can deploy multiple warheads, would be the heart of the system. The report identifies a range of technologies that could become capable of destroying missiles — including X-ray lasers, chemical, excimer and free electron lasers, particle beams and kinetic energy

devices. Congress would be asked to spend $27,000 million on research between fiscal years 1985 and 1989. Deploying the first layer of a multi-layered defence system by the year 2000 would cost about $95,000 million.

The interagency report is based on two separate studies. One, a technical investigation headed by James Fletcher, a former administrator of the National Aeronautics and Space Administration (NASA), contains few surprises. It emphasizes that all the necessary technologies are in an early stage of development and that years of research will be needed to turn them into practical weapons. The other, a strategic policy analysis headed by Fred Hoffman, director of a private think-tank, is likely to be more controversial. It claims that development of the system will not, in the short run, conflict with the 1972 Anti-Ballistic Missile Treaty; merely carrying out research for the new system will give the United States a strategic advantage by "confusing" the Soviet Union and forcing it to invest in expensive counter-measures.

A similar view was expressed earlier this month by George Keyworth, the President's science adviser. Acknowledging that it would take up to six years before the United States could choose between the most promising technologies, Keyworth maintained that an early demonstration of technical progress — such as a multi-megawatt pulsed laser with a beam corrected for atmospheric distortion — might be enough to persuade the Soviet Union to soften its negotiating position on arms limitation.

At a press conference last week, President Reagan said he had not yet read the interagency report. Administration officials, however, confirmed privately that the details published in *Aviation Week* were accurate. The report calls for early demonstrations of the effectiveness of infrared chemical lasers, ground-based excimer lasers and shorter wavelength chemical lasers. Other demonstrations would focus on the tracking and targeting of ballistic missiles by means of long-wavelength infrared sensors and high-altitude aircraft.

Peter David

High-energy physics

US plans take shape

Washington

THE US Department of Energy (DoE) has officially cancelled the Brookhaven National Laboratory's colliding beam accelerator (CBA, also known as ISABELLE) and is shifting most of the money for it in this year's budget to research and development on the proposed 20 TeV proton–proton collider, as recommended by its High Energy Physics Advisory Panel (HEPAP).

The HEPAP recommendation, made months ago (see *Nature* **304**, 105; 1983), marked the *de facto* end of the project, a victim of the success of the European Organization for Nuclear Research (CERN) with its SPS facility and of several years of technical and management problems at Brookhaven. Representative William Carney (Republican, New York), who represents Brookhaven's congressional district, could not resist using DoE's official decision, however, as an opportunity for firing some parting shots.

At a hearing last week, he grilled Dr Jack Sandweiss of Yale University, the chairman of HEPAP, over the panel's "inconsistency" in recommending the cancellation of a project that it had in past years repeatedly endorsed. He also asked Dr Sandweiss how he was going to "explain to the taxpayers" the $200 million that has already been spent on the Brookhaven project.

Carney presented the committee with a slide presentation on CBA of which high point, the was a LANDSAT photo of Long Island where the only identifiable man-made feature, he said, was the CBA site. Sharp-eyed reporters, however, claimed to be able to see the Long Island Expressway as well.

Dr Alvin Trivelpiece, DoE's director of energy research, said that $18 million of the $23 million in operating funds appropriated for CBA in the current budget will be redirected to research and development on the "superconducting super collider", the 20 TeV machine endorsed by HEPAP. Brookhaven will receive $10.1 million of that. Brookhaven will also receive some $4.3 million to cover the costs of closing down CBA and $700,000 to upgrade its Alternating Gradient Synchrotron (AGS).

DoE expects to obtain approval from the House and Senate Appropriations Committees to redirect these funds. A specific appropriation request for the construction of the super-collider will be necessary, and that will come only when there is a detailed design plan for the machine.

Research and development will follow a four-year plan laid out in September by a HEPAP subcommittee chaired by Dr Wolfgang Panofsky, director of the Stanford Linear Accelerator Center, and the chief aim of the research will be to determine the optimal cost trade-off between tunnel length (which will be at least some tens of miles long) and magnet field strength.

Although Carney is publicly deriding the super-collider as a "dream machine" and lamenting the loss of the taxpayer's investment in CBA, his aides say he has no plans to block the new plan. They say that Carney's major concern now is to persuade DoE to commit itself to finding an alternative use for the CBA tunnel.

One increasingly likely prospect would be to link the Alternating Gradient Synchrotron to the CBA tunnel to create a relativistic heavy-ion collider, a project that Trivelpiece estimates would cost $7–8 million. The first step, now under consideration by DoE, would use $5 million appropriated in the current budget for CBA construction to link Brookhaven's tandem van de Graaff accelerator with AGS to create a heavy-ion capability.

Carney's immediate need may be satisfied, but in the long run what will matter more is whether the new plan can keep Brookhaven on its toes.

Stephen Budiansky

0028-0836/83/430752-01$01.00

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Antarctica

Treaty goes to wider forum

Canberra

THE members of the Antarctic Treaty now seem reconciled to the prospect that the future of the treaty will be debated by the United Nations (UN) General Assembly, most probably towards the end of November. The underlying issue is whether exploration and possibly exploitation of Antarctica should rest with the signatories of the Antarctic Treaty or be thrown open to a larger group of states.

At the meeting of the non-aligned nations in Delhi, India, earlier this year, there were several expressions of discontent about the alleged exclusiveness of the Antarctic Treaty. The issue was raised in the UN General Assembly on 14 October by the Deputy Prime Minister of Malaysia, Dato Musa Hitam, who denounced the treaty as "restrictive and exclusive". Malaysia's contention is that Antarctica, like the deep ocean bed, should be regarded as part of the "common heritage of mankind" and should be made accessible to all nations irrespective of "their economic and scientific development and capabilities".

Malaysia's objections have to some extent been undermined, however, by the admission of India and Brazil as full members of the Antarctic Treaty on 12 September, in time to take part in the regular consultative meeting of the treaty members held in Canberra from 13 to 27 September. The two countries have qualified by means of scientific expeditions to Antarctica, India in the past two seasons and Brazil in 1982–83.

In a new departure, representatives of states that have signed the treaty but which have not been admitted as "consultative parties" on the basis of their demonstrated interest in Antarctica were present in Canberra as observers. Conspicuous among these was China, which has established a national committee for Antarctic research, which is thinking of applying to join the Scientific Committee for Antarctic Research (SCAR) organized by the International Council of Scientific Unions (ICSU) and which seems to seek the status of a consultative party.

The views of the existing parties to the treaty on the forthcoming UN debate are probably accurately represented by the statement by Mr Bill Hayden, Australia's Minister for Foreign Affairs, at the opening of last month's meeting. The Antarctic Treaty is "alive and well", he said, and any attempt at renegotiation would "introduce uncertainty and confusion into a region of hitherto unparalleled international cooperation".

The most likely outcome of the first UN debate next month is that the secretary-general will be asked to carry out an investigation and to report back next year. What happens afterwards is an open question.

Vimala Sarma

Correction

MR Barrie Jones, Minister of Science and Technology in the Australian Government, denies that he had sought opinions on the future of the Australian National Animal Health Laboratory "from private individuals and the bureacracy without discussion with CSIRO", as reported in *Nature* on 22 September (p264). In a telegram to our Canberra correspondent, Mr Jones says "This is not true". His statement is gladly accepted. — Editor, *Nature*

An exhibition at Heslington Hall, University of York, celebrates the centenary of the animal artists Charles Maurice and Edward Julius Detmold. "Cockatoo and Monkey", shown above, was painted by Edward Julius in 1924. The exhibition is in York until 18 November, at the University Art Gallery in Nottingham from 25 November to 17 December and at the British Museum (Natural History) in London from 6 January to 4 February 1984.

UK higher education

Public sector's brownie points

WHILE British universities consider how they might fit in more students at no extra cost, non-university colleges have been told they may loosen their belts a little. Sir Keith Joseph, Secretary of State for Education and Science, is proposing to give polytechnics and colleges of advanced further education £20 million over and above what they had been planning to spend next year. Their number of students will be increased only slightly above the total implied by a major planning exercise by the National Advisory Body for local authority higher education (NAB).

The extra cash seems to be a reward for good behaviour, as defined by the Department of Education and Science. NAB had been asked to plan for the academic year 1984–85 on the basis of a level budget. Its response was, broadly, to maintain student access and to complain about the decrease in the unit of resource (education planning jargon for expenditure per full-time student equivalent). This course was the opposite from that taken two years ago by the University Grants Committee.

Now the strategy seems to have paid off. NAB's student total, about 257,000, was more than the Department of Education and Science (DES) had hoped for and was within an ace of its notional target. NAB has now been asked to find places for an extra 2,000 students next year, the direct cost of which will be only £4 million. The increase, says DES, will ensure orderly rationalization and preserve the 11.7:1 staff/student ratio that is recommended by the department's inspectors. Total expenditure in the sector will now amount to £580.5 million.

Despite the tonic of the extra £20 million, it will still be necessary for polytechnics and other colleges to lose "significant" numbers of lecturers, and NAB's overall objective remains to concentrate resources into the major institutions. Details will be finally agreed next month. The chief departure from its original plan is that NAB has partially backed away from its policy of diverting resources away from South-East England.

Without the latest increase for the local authority maintained sector, its unit of resource would have fallen by rather more than 13 per cent in two years. By effectively reducing the differences in support for universities and other colleges, DES will have managed to contain the demographic peak in student demand in 1984 and 1985 at little extra cost: the £20 million which is now expected to go the non-university sector is comparable with the savings made if, as expected, universities manage to comply with a request from the University Grants Committee to find space for an extra 5,000 students next year.

Tim Beardsley

0028-0836/83/430753-01$01.00

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Acid rain

Congress makes the running

Washington

POLICY-making on acid rain is once again in tatters in the United States. Since William Ruckelshaus was appointed last spring to head the Environmental Protection Agency and asked by President Reagan to tackle the acid rain problem "head on", he has made the development of an acid rain policy a personal commitment. Throughout the summer he held meetings with interested groups and received a substantial amount of scientific advice. But when he recently presented two detailed proposals for reducing sulphur dioxide emissions as a first step towards solving the problem, he met head-on opposition from the Reagan Administration. The immediate consequence was an embarrassing meeting last week with Canadian officials who had earlier been assured that a US policy on this issue, which is of immense concern to Canada, would at long last be announced. The long-term consequence may be that the administration will lose the initiative on acid rain policy to Congress.

Ruckelshaus's proposals, presented last month to the cabinet council on natural resources, are modest enough. One would reduce sulphur dioxide emissions in four mid western and north eastern states by 3 million tonnes a year, about half present amounts. The second would make similar reductions in two mid western states, giving a total reduction of 4.4 million tonnes.

The National Academy of Sciences this summer concluded that a nationwide reduction in sulphur dioxide emissions would bring about a proportionate reduction of total acid deposition but that the benefits in specific regions of specified reductions elsewhere could not be predicted with any certainty. Total sulphur dioxide emissions in the eastern United States amount to approximately 18 million tonnes a year.

The academy's warning notwithstanding, the Ruckelshaus proposals appear to have relied heavily on computer modelling of regional effects, and were designed with the specific goal of alleviating acid deposition in New England and the Adirondack region of New York, areas that contain many sensitive lakes.

Ruckelshaus has encountered opposition not only from the administration but from environmentalists and conservationists, who have also relied on computer modelling to denounce them as inadequate. A broad coalition of these organizations wrote to President Reagan to complain that neither the two areas singled out in the Ruckelshaus plan nor four other critical areas — the Smoky Mountains in the south-east, the boundary waters region in northern Minnesota, western Pennsylvania and eastern Canada — would benefit to the extent required to prevent damage from acid deposition. They called for a nationwide reduction of 50 per cent in sulphur dioxide emissions.

Several members of the Reagan cabinet objected on quite different grounds. After last week's meeting with Canada's environment and foreign affairs ministers in Halifax, Nova Scotia, Ruckelshaus admitted that he had wanted to present a US plan at the meeting but that cabinet officials who "brought different perspectives" to the issue had blocked action on his proposals. His chief opponent is reported to be David Stockman, director of the Office of Management and Budget.

There seem to be four points of disagreement. First, there is a dispute about the validity of the scientific evidence. How the programme would be administered is unclear, while the administration seems to be alarmed that one regional constituency or another is certain to be affronted by proposals to reduce sulphur dioxide emissions by the use of low-sulphur coal or the installation of expensive scrubbing equipment. The overriding anxiety, however, is the cost of the Ruckelshaus proposals — and their consequences for electricity tariffs.

Canada's environment minister, Charles Caccia, is openly chagrined by Ruckelshaus's setback. He said last week that he had been encouraged by an earlier meeting with Ruckelshaus on 8 September but that now Canada was becoming "impatient". One obvious difficulty for the United States is that during the Carter Administration, the United States committed itself to negotiating with Canada a solution to acid rain and transboundary pollution in general. The Reagan Administration, however, has been moving very slowly and has effectively broken off negotiations. The only consolation offered at last week's meeting, an agreement which aimed to reduce phosphorus pollution in the Great Lakes by 15 per cent, failed to mollify the Canadian negotiators.

Congress, which has put off work on acid rain legislation since Ruckelshaus's appointment, is now moving quickly. The Senate Environment Committee, whose chairman, Robert Stafford (Republican, Vermont), has frequently spoken out on the need for new controls on acid rain precursors, has arranged to hold hearings next month with the intention of devising legislation for the control of acid rain.

Stephen Budiansky

East-West cooperation

Bulgarian link shows promise

MR John MacGregor, Minister of State at the British Ministry of Agriculture, Fisheries and Food, who returned recently from Bulgaria, is cautiously optimistic about the possibility of expanding cooperation between the two countries on agricultural research. Such research has been taking place for some years under a general bilateral agreement on trade and technology, and earlier this year, a joint British-Bulgarian-Romanian seminar was held in Britain on modern agricultural methods.

The history of such East-West collaboration has so far been far from happy. For example, after a recent series of seminars on energy-saving technologies for agriculture involving British and Romanian scientists the British specialists felt that the Romanians were insufficiently advanced in this field to make a positive contribution to joint studies.

Bulgaria, however, has one of the best developed agricultural sectors in the socialist bloc and considerable efforts have been made over the past three decades to make agriculture more productive. Such programmes have occasionally reflected the zeal of one group of planners rather than the "integrated" approach to the economy urged by Communist Party directives — for example, a major investment in the tobacco industry was announced earlier this year at the same time as the Ministry of Public Health was promoting an anti-smoking campaign.

For the most part, however, Bulgarian agricultural research concentrates on the well established fields of crop selection and the study of plant diseases. Fortunately, Bulgaria was spared the worst effects of Lysenkoism; although this theory was for a short time a necessary part of university biology courses, it never really affected practical work. The results, for the most part, have been steady rather than spectacular. Recent innovations include the development of a golden tomato with beta-carotene content five times higher than the ordinary red varieties and the breeding of a new silage crop derived from the wild legume *medanka,* which, it is claimed, not only yields up to 10 tonnes to the hectare, but helps to combat sterility in ruminants, can grow on marginal soils up to 1,300 m above sea-level, and (with the aid of bees) yields 600 kg of honey to the hectare.

Bulgaria's main interest in any exchange with the United Kingdom centres on the high productivity of British agriculture. The main problem, now, is logistics — however keen the British team may be, the parties to any agreement must be individual agricultural institutes, not the ministry as such. And it is precisely on the difficulty of cooperation between individual bodies on the one hand, and a centralized state apparatus on the other, that so many East-West cooperation agreements become bogged down.

Vera Rich

0028-0836/83/430754-01$01.00

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US biotechnology

Living with regulation

Washington

A SCORE of biotechnology companies received mixed signals when their representatives met here last week to discuss the regulatory outlook for their industry. Federal officials told them the government was not interested in "brazen over-regulation" and Representative George Brown (Democrat, California) said biotechnology had many friends on Capitol Hill. But the companies were also warned that they will soon face a new group of regulatory hurdles and that the ultimate success of their industry depends on convincing the public of its safety.

The major focus of regulatory action in the coming months will be the Environmental Protection Agency (EPA), which has declared its intention under the Toxic Substances Control Act (TOSCA) to regulate the development of new genetically engineered substances. The agency's acting assistant administrator for pesticides and toxic substances, Don Clay, told industry leaders that EPA was making no presumption that recombinant DNA substances were toxic; they would be looked at in the same way as other substances. But he confirmed that EPA intends to exercise great caution before approving the release of recombinant microorganisms.

In an unwelcome echo of the lawsuit brought by environmental activists against the controversial frost-retardation experiment planned at the University of California, Berkeley (see *Nature* 13 October, p.564), Clay said it is possible that genetically engineered microorganisms may have adverse environmental consequences akin to those of the kudzu weed and the gypsy moth. EPA is therefore working on a set of questions that manufacturers will be required to resolve before the general release of such substances could be approved.

The list will include questions about the ability of organisms to survive under different conditions, to reproduce, to travel, to transfer genetic traits to natural organisms and to develop resistance against control agents. EPA would also want to know how the population of an engineered organism in the environment could be monitored and controlled. The questions will be published as guidelines early next year.

The meeting was convened by the Industrial Biotechnology Association (IBA), the fledgling consortium of companies set up in 1981 to promote the interests of the industry nationally. Privately, members of the association expressed doubts about EPA's competence in drawing up regulations for biotechnology products. While the Food and Drug Administration (FDA) received high marks, EPA was criticized for having too few scientists with strong credentials in biotechnology. Clay, however, told the meeting that EPA has started talks with other agencies to see whether it would be possible to pool the federal government's expertise in biotechnology regulation in a single interagency committee.

Representative George Brown told IBA that EPA's approach to biotechnology would be a "major decision point" for the industry because it would set the tone for other government agencies and deal with fundamental unresolved issues, such as the definition of genetically engineered organisms and "new" substances. Congress, he said, is unlikely to adopt legislation on biotechnology in the near future, but he advised IBA to act early to educate the public and eliminate the "more irrational" pressures on Congress.

A similar warning was delivered by Nanette Newell, a member of the congressional Office of Technology Assessment and director of a forthcoming study of American competitiveness in biotechnology. Claiming that American industry is more liable than its competitors to suffer as a result of public perceptions, she urged IBA to step up its efforts to improve public understanding. The lawsuit brought by anti-DNA activist Jeremy Rifkin is an example of how a small number of people could generate a great deal of public concern, she said.

IBA itself is divided on how to deal with the Rifkin lawsuit. A proposal that the association submit an *amicus* brief on the side of the National Institutes of Health was rejected. One problem is that IBA still has fewer than 30 member companies and is short of funds. But some members are said to believe that it would be a mistake to transform a dispute between Rifkin and the federal government into a dispute in which the industry itself took too visible a part.

Peter David

Information technology

Selling British software

BOTH government and industry are urged to take better account of the value of tradeable information, in a report from the British Government's Information Technology Advisory Panel. The panel says that responsibilities in this area are "fragmented", as are the efforts of industrial companies, and proposes institutional changes to remedy the situation. Chief among these is the suggestion that a government minister should be appointed with overall responsibility in the area.

The panel's central thesis is that hardware advances are eroding traditional barriers between sectors of the information industry, defined by the panel as including everything from scientific publications to video entertainment. It complains that while government initiatives and industry's efforts have been devoted to hardware, opportunities in software development have been neglected.

But apart from this general exhortation, the panel makes few detailed recommendations. It stresses the need for more cooperation between companies and calls for interdepartmental government machinery to improve coordination. It fails to tackle the specifics of what it sees as the most important single task of government in the field: the speedy introduction of up-to-date copyright legislation.

The general message is nevertheless clear. Britain, according to the panel, has the potential to become a world leader in tradeable information, and the market is expected to grow rapidly. As things stand, however, there is no way of finding out whether or not Britain is a world leader, because there is an almost total lack of reliable information on information. The panel concludes that the lack of economic statistics on the tradeable information sector is a "grave defect".

The panel repeats the familiar comment that the main responsibility for development in the sector should rest with private industry, but also adds the equally familiar rider that there is much that government could do to facilitate the process. One major stimulus would be the publication of more government information in machine-readable form, which would encourage industry to set up suitable information networks. The means by which government receives information should also be kept under review to promote the widest possible use of machine-readable data. The panel identifies the provision of government support for the development of advanced software for searching databases as an important priority.

Tradeable information is not, however, to be reserved exclusively for hard-headed businessmen, and the panel would like to see the research councils sponsor further academic study of the economic potential in this field. The panel has a further note for those already active in supplying information, publishers of scientific journals included. They should, it says, examine their current activities closely — the development of scientific journals could before long start to limit the market for printed matter. Fortunately, perhaps, for those publishers, libraries do not yet commonly subscribe to electronic information networks, partly perhaps because of the cost. The slow take-up of British Telecom's Prestel system is attributed to a failure to recognize that the public will not pay simply for information which is at present free just because it is presented on a screen.

Tim Beardsley

0028-0836/83/430755-01$01.00

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Salyut-7

Cosmonaut crisis exaggerated

REPORTS in the US journal *Aviation Week* that the Soviet orbital spacecraft Salyut-7 suffered a rupture in a main oxidizer line at the beginning of September have triggered speculation that this could put at risk the lives of cosmonauts Vladimir Lyakhov and Aleksandr Aleksandrov. These suggestions (which did not have the backing of the *Aviation Week* "analysts") have been formally denied by Soviet space planners. The launch last week of the cargo ship Progress-18 was announced in terms which made it clear that no early return to Earth was contemplated. This replenishment craft carried fuel supplies for Salyut-7, rations for the crew, and also materials for further experiments, Tass said last week.

But the Tass statement has not ended speculation in Western newspapers. There are said to be two main dangers threatening the cosmonauts. First, if the Salyut is still losing fuel, it might become impossible to make essential orbital corrections. There is also the problem of the cosmonauts' return to Earth. Transport to and from the Salyut stations is by the Soyuz two or three-person transport craft. For longer flights, these craft have the major disadvantage that after about 115 days in orbit, the propellant tanks, batteries and some of the sensitive electronics are considered to be too unreliable to ensure a safe return to Earth. If the cosmonauts are to remain in space longer than three months, they will need a new spacecraft for their return trip.

This, in normal conditions, presents no great problem. The Salyut has a docking unit at each end, so that it is perfectly feasible for a second Soyuz to dock with the complex while the original transport craft is still in place. This replacement is normally effected by sending up a short-term guest-crew before the safe life of the original Soyuz has expired; the guests then return to Earth aboard the old Soyuz, leaving the new one coupled to the Salyut.

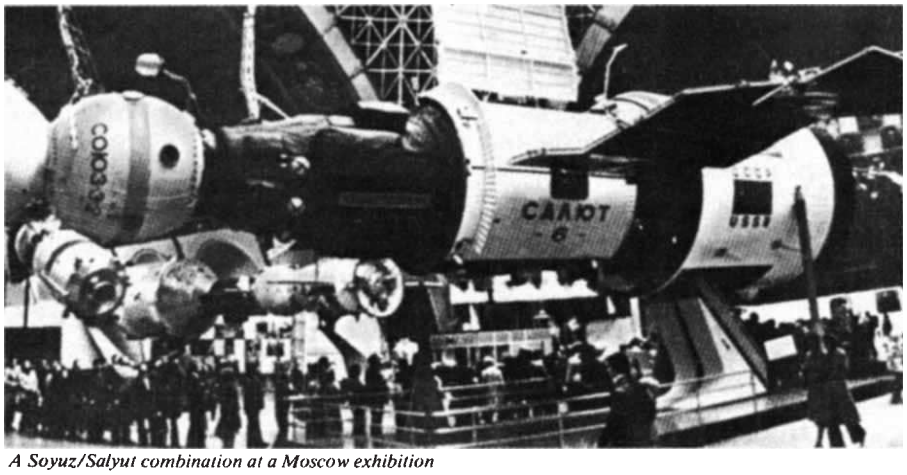
Unfortunately, the replacement Soyuz that should have been launched at the end of September blew up on the launch pad (its crew of three only narrowly escaping with their lives). Since Soyuz-T-9, which carried Lyakhov and Aleksandrov up to Salyut-7, is now almost four months old, they will need a new craft in which to return.

This should, in itself, pose no difficulties. Following other problems in the past, notably the docking failures of Soyuz-25 and 33, the Soviet manned programme has previously been temporarily suspended. After the recent even more serious incident, the space planners may well wish to make a thorough investigation of what caused the explosion before attempting another manned flight. If, however, it becomes imperative to bring Lyakhov and Aleksandrov back to Earth, it would still be possible to send up an unmanned Soyuz to dock automatically with Salyut.

The idea put forward in some newspapers that the Soviets may have sent up Progress-28 to keep the cosmonauts alive until they can be rescued by the US shuttle in November is somewhat fanciful — such a rescue would, theoretically, be possible but replenishment by Progress is a routine feature of long-term stays on Salyut and should not be interpreted as a symptom of alarm.

Indeed, the only definite signs of concern for the safety of the cosmonauts have come not from the engineers but from Soviet physicians. Cosmonauts on previous long-term missions have shown a significant decline in activity and efficiency during their fourth month in space. This time it is hoped to counter this by a major change in the experimental programme. During the first three months, the cosmonauts concentrated on geophysical observations, but from now on they are to work on the production of new semiconductor crystals and biologically pure substances. Furthermore, to improve their physiological well-being and to overcome the sense of isolation from Earth reported by their predecessors, Lyakhov and Aleksandrov are woken each morning by recorded bird-song.

Vera Rich



A Soyuz/Salyut combination at a Moscow exhibition

Spanish universities

Autonomy on trial

Barcelona

AFTER a marathon two-day debate, the Law for University Reform was approved by the Spanish Senate before the summer recess and is now taking effect. The Socialist majority in both houses wanted quick approval and the bill passed through Parliament without any major change; the Senate did not alter a single word. A previous bill, the so-called Law for Autonomy of the University, remained in Parliament under four successive ministers of education, and suffered many changes before being withdrawn by the last centrist government a year ago.

The new Law for University Reform is intended to provide a general framework for higher education in Spain. It defines the university as a public service, and higher education as the right of everyone who wishes to pursue it. The law is intended to help Spanish universities to achieve their objectives of "scientific development, the education of professionals and the spreading of culture".

Under the new law, university institutions will have much greater autonomy in both economic and academic matters. The universities will now have the right to change their curricula, to select their own professors, to decide their own internal statutes and to administer money from the state and from their own resources. All these changes are intended to encourage diversity and competition among Spanish universities. Most of these measures have long been sought by university authorities.

One part of the law that has surprised many scholars has been that relating to professors. Contrary to previous socialist party policy, the law does not change the traditional status of of professors as civil servants. Only a limited number of visiting and associate professors with fixed-term contracts will be allowed. Professors will be divided into "titular" professors and *"catedrático"*, both tenured but with no specifically different functions. To become a full professor (*catedrático*), three years experience as a titular professor will be necessary. The system of selection of teaching staff has also been modified, with a procedure based on candidates' *curricula vitae* instead of the classical examination procedure (*oposiciones*). All the teaching staff now under contract will in the next four years have an opportunity to achieve a permanent position through a special procedure. Spanish scientists working abroad can also take part under certain conditions.

There has been a rush to introduce the changes in time for the academic year now beginning. Not until the system has been running for at least a year will the real effects of the law on Spanish universities be known.

Pedro Puigdoménech

0028-0836/83/430756-01$01.00

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UK public enterprise

Reprieve by delay and default

THE British Technology Group (BTG) seems still to be in a state of some confusion over the future conduct of its affairs. In September, BTG suffered the indignity of having its "first option" on the exploitation of much publicly-funded research described as a "mistake" by the Prime Minister, Mrs Margaret Thatcher, who said that the right would be rescinded. But BTG is now telling all callers that, for the time being, it is business as usual.

BTG is an unofficial liaison of two earlier bodies, the National Research Development Corporation (NRDC) and the National Enterprise Board (NEB). On 30 September, Mr Cecil Parkinson, then Secretary of State for Trade and Industry, announced that BTG would in future concentrate on the traditional NRDC role of a technology transfer agent, while NEB would continue to dispose of its investments in high-technology companies. NRDC has asked for more detailed information on financial priorities, but no clear policy has emerged from a 15-month government review. Now NRDC's chairman, Sir Freddie Wood, has announced his intention to retire shortly and BTG will not be able to draw up detailed plans until a successor has been named.

The task of Sir Freddie's successor will not be made any easier by financial results published last week in NRDC's annual report. The declared objective of the government is for BTG to become self-financing, but NRDC's profits fell from £10.4 million in 1981-82 to only £2.3 million in 1982-83. The fall is due mainly to amortization of investments made several years ago, after a critical review of projects which were failing to live up to expectations. Income from licences actually rose, although patents on BTG's main money spinner, the cephalosporin antibiotics developed at the University of Oxford, are starting to expire and income from them will fall substantially over the next few years. Income from pyrethrin insecticides will also decline.

BTG's most ambitious accomplishment last year was the formation, in collaboration with private investors, of the Agricultural Genetics Company. The company has an agreement with the Agricultural Research Council to develop research from its laboratories with commercial applications but the venture is not expected to show a profit for several years.

Mr Parkinson's successor at the Department of Trade and Industry is Mr Norman Tebbit, who has previous experience in the department and is a keen advocate of the more liberal climate for innovation that the government hopes to foster. But, quite apart from the requirement for BTG become self-financing, there are constitutional difficulties to be overcome. Chief among these is the fact that BTG would not be allowed to act a commercial patenting service, and there are worries that some universities may be unwilling to take on an entrepreneurial role themselves and seek arrangements with commercial agents.

Although there is some frustration among BTG executives about the government's apparent indecision, BTG has become more responsive to laboratory developments. During the past year, a university coordination unit has been expanded and a database of inventions has been established for use by industry and researchers. BTG will also shortly announce the appointment of four regional officers to act as the group's eyes and ears in research institutions. As for the winddown of the old NEB role, BTG has for some years been disposing of its investments and is now trying to find sources of private capital that would allow it to extricate itself from Inmos, the British semiconductor manufacturer. But such operations take place overnight only at a loss.

Tim Beardsley

Glory game

THE 1983 slogans for the revolution day parades in Moscow on 7 November contain a certain note of criticism of Soviet science.

Last year, the relevant slogan read "Soviet scientists! Improve the effectiveness of research! May the alliance between science and production become stronger! — Glory to Soviet science!"

This year the "glory" has departed, and the scientists are apparently not producing the results that industry requires. The slogan reads: "Soviet scientists! Improve the 'resultivity' of your work! Actively cooperate in implementing the achievements of science in production".

Nor is this a matter of mere general exhortation. The party central committee last week passed a resolution on the work of the Urals Centre of the Soviet Academy of Sciences suggesting that while doing much valuable work in fundamental and applied research and in training a new generation of scholars, the centre is falling behind on its main task of "improving the efficiency of public sector production, increasing productivity and the economic and social development of the region".

Research efforts must be devoted, the resolution said, to "key fundamental and applied research" dictated by the development needs of the "productive forces of the Urals". Measures for the "fuller and more rational use of the scientific potential" of the region must be implemented. And the presidium of the centre is "advised" to "improve decisively" its coordination of the work of Ural research establishments.

Vera Rich

Software business

UK Logica goes public

THE London Stock Exchange, easily adaptable except to the British Government's suggestion that commission rates for brokers might be flexible, is about to make a market for the largest British software company to go public. Logica plc, founded 14 years ago by five software engineers but which insists that it is not a mere software house, is about to sell off nearly 30 per cent of its shares to the public in the next few weeks.

Part of the interest of this development is that Logica has been one of the more conspicuously successful British companies operating in a novel area of technology in recent years. The accounts show that turnover has grown exponentially at 33 per cent a year. Profits when smoothed out (1980 seems to have been a bad year because of the operations of Logica's Dutch subsidiary) have grown almost as quickly, and amounted to £3.346 million in the financial year to the end of June.

Unlike other British companies, Logica seems to have had ready access to venture capital. Between 1979 and 1982, the government-backed National Enterprise Board helped Logica with loans of £1.8 million and with an equity investment sold at a profit more than a year ago for £3.4 million to a group of financial institutions.

One striking feature of the company's ownership is that even after the public offering now planned, 38 per cent of Logica will be owned by 220 of its 1,600 employees. The company's prospectus says that this is part of a deliberate policy appropriate to a business "which relies heavily on the skills of the employees". Dr Philip Hughes, chairman of the company and one of its founders, said last week that Logica last year recruited 130 university graduates, in which respect it is as important a user of technical skills as even the largest British companies.

Logica is openly boastful of the reputations of its clients, which include major oil companies, supermarket chains and even computer manufacturers and electronics companies. Since 1979, the company has also been a manufacturer of word processors and personal computers. For most of its 14 years, Logica has earned getting on for half of its turnover overseas.

The offer for sale follows the present fashion on the London Stock Exchange of inviting tenders for the purchase of stock. The minimum price of £1.40 a share values the whole company at £49 million. The company is declining to forecast profits for the current year, but has been persuaded to break with its past practice of not paying dividends to its shareholders by promising those who subscribe for its shares a return of roughly one per cent during the year ahead. □

0028-0836/83/430757-01$01.00

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Animal rights or welfare?

SIR — In the leading article "Animal rights nonsense" (*Nature* 13 October, p.562), your writer seeks to destroy the concept of animal rights, but appears to have a distorted idea of what philosophical theories of rights actually entail. He seems to believe that espousal of animal rights necessarily means the belief that animals have a diverse set of absolute and inalienable rights. In fact, very few rights theorists (notably some pacifists) would claim that this is true even of human beings. Few, for example, would deny that other humans have a general right not to be tortured; equally, few would deny society the right to use torture to reveal the site of an atomic bomb beneath London. Professor Bernard Rollin expresses the theory of animal rights succinctly, "if one takes the position that the right to life is not absolute in the case of humans, but that strong moral reasons must be given in defence of any violation of that right, one must take a similar position *vis-à-vis* animals".

This kind of principle does not mean that in a genuine conflict of serious interests one is not entitled to hold that (generally speaking) a human life is more valuable than that of a non-human animal. It does mean that the animal's rights ought not to be infringed for frivolous, trivial or avoidable reasons. I personally believe that temporary pleasure of the palate, vanity, commercial profit and the cure of readily preventable or minor illness represent such unacceptable reasons.

Your writer's second premise, that only those with duties can have rights, would not appeal to most defenders of human rights, as it clearly rules out rights for mentally-ill, severely retarded or very young humans. I doubt whether any civil rights campaigners would accept his view that subordinates can expect no rights unless they comply with all rules laid down by those in authority, however arbitrary. If, however, he really means the sort of tacit agreements which enable us to live together without violent conflict, surely the dog passes this test with flying colours. Dogs may not reliably sit on command, but at least your staff member was not bitten during his experiment in animal responsibility.

Comparison of animals and children eliminates the objection that, if animals have rights, these must be identical to those of adult humans. Most of us would agree that young children have rights, but this does not mean that we consider them competent to refuse life-saving treatment, one of the rights of adult humans. Clearly the same holds good for animals.

Finally, he argues that concern should be given to animal welfare, not rights. The trouble with this attitude is that it is then all to easy to see reforms in our treatment of animals as optional extras, acceptable if they do not cost very much, and even more acceptable if they actually benefit us in the long-run anyway. The language of rights is important because it makes the point that it is *obligatory* for us to treat animals as objects of moral concern.

ROSEMARY RODD
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Cambridge CB1 2LL, UK

Clone availability

SIR — A recent News and Views article[1] states that investigators from Cold Spring Harbor and Massachusetts Institute of Technology have not "yet been able to test the transforming genes of chicken and now human B-cell lymphomas" which several colleagues and I have described. According to this article, this is because the cloned genes are "unavailable". The implication is that we have refused to supply these clones. On the contrary, we have supplied the chicken *Blym-1* clone to all laboratories (16 as of 20 September) who have requested it since publication of its isolation[2]. Similarly, the newly published[3] human *Blym-1* clone is now available to the scientific community. Neither of these clones has been requested by the investigators or laboratories referred to in your article.

GEOFFREY M. COOPER
Dana-Farber Cancer Institute,
Boston, Massachusetts 02115, USA

1. *Nature* **305**, 470 (1983).
2. Goubin, G., Goldman, D.S., Luce, J., Neiman, P.E. & Cooper, G.M. *Nature* **302**, 114-119 (1983).
3. Diamond, A., Cooper, G.M., Ritz, J. & Lane, M.A. *Nature* **305**, 113 (1983).

The Yogi in *Nature*

SIR — You will surely agree with me that anybody who claims that details of grand-unification theories are "available" in some ancient script (albeit, alas, in cryptic form) is a joker. I was rather upset to find an advertisement by a clown like the guru Maharishi Mahesh Yogi in a serious journal such as *Nature* (8 September). Why was this advertisement, in my opinion a serious corruption of *Nature*'s image, accepted for publication? If it was because advertisements are paid for and *pecunia non olet*, then *Nature* has embarked on a dangerous and counterproductive course. (Can we look forward to similar advertisements by scientologists and creationists or for von Däniken's latest brainwave?)

I fear that you will be used. These clever fanatics will boast on the very fact that some of their lunacies appeared in a respectable scientific journal. That it occurred in an advertisement is for them irrelevant. That is why this type of advertisement should be refused. The guru must be denied the slightest trace of a suggestion of being taken seriously in a scientific journal. An advertisement for an astrologer is out of place in an astronomy or astrophysics journal and similarly, an advertisement of people who use quasi-scientific terminology to give their dogmas an air of scientific legitimacy is out of place in *Nature*.

E. J. ZUIDERWIJK
European Southern Observatory,
Casilla 16317, Santiago 9, Chile

Hepatitis B vaccine

SIR — We would like to re-establish the truth concerning our company's policy on the production of hepatitis B vaccine (*Nature* **304**, 297; 1983). Our production process calls for the sole use of HB-E-negative plasma. No plasma of whatever geographical origin has ever been used in the production of HEVAC B without passing the HB-E-negative control. Therefore the information given about Institut Pasteur Production's "broken promise" is wrong and is based on false information.

Y. GARNIER (President)
Institut Pasteur Production,
3 bd Raymond Poincaré,
Marnes-la-Coquette, France

Nuclear deterrents

SIR — The British Government's deliberate vagueness on civil defence planning may well be cause for ridicule but the recent disclosure that there are serious deficiencies in the Home Office computer model for predicting the number of casualties in the event of a nuclear attack (*Nature* 6 October, p.459) must be cause for concern. These deficiencies when taken together suggest more of a deliberate attempt to minimize casualty estimates than mere incompetence. Perhaps the government does not wish to undermine confidence in the nuclear deterrent by suggesting that if deterrence fails all will be lost.

Your editorial advice to the nurses (and other professional groups) on how best to argue what would be the most probable form of nuclear attack would have been relevant 20 years ago before the deployment of accurate counterforce nuclear weapons. In the 1960s nuclear weapons were only accurate enough to destroy large targets such as cities but not small protected targets such as missile silos. Nuclear retaliation was invulnerable to attack, so a few nuclear demonstration shots might not have precipitated a full-scale nuclear exchange. But deployment of accurate counterforce weapons, by threatening to destroy the enemy's missiles in their silos, lowers the threshold for nuclear war.

In times of crisis the pressure to strike first will be enormous. Deployment of cruise and Pershing missiles in Europe will tie NATO's strategy of flexible response to the strategy of counterforce. Demonstration shots would bring the card house down.

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Final crop of 1983 Nobel awards

The Nobel committees have awarded this year's physics prize to Professors W. A. Fowler (Caltech) and S. Chandrasekhar (Chicago) and the chemistry prize to Professor Henry Taube (Stanford).

THE 1983 physics prize is shared between Dr S. Chandrasekhar (see following page) and Professor William A. Fowler, until his recent retirement professor of physics at the California Institute of Technology, Pasadena. Fowler's share of the prize is awarded for his experimental and theoretical studies of nuclear reactions relevant to the theory of nucleogenesis, one of the outstanding landmarks of the 1950s.

By background a nuclear physicist, Fowler has since the Second World War been the one chiefly responsible for making Caltech a prolific source of fruitful applications of nuclear physics in other fields, many with an astrophysical or cosmological bearing. His distinctive contribution to the successful solution in 1957 of the problem of nucleogenesis stemmed from his careful experimental measurements of many of the cross-sections of nuclear reactions thought to be crucial for energy production and the synthesis of heavy elements in stars. This problem was the central theme of Fowler's long spell as director of the Kellogg Laboratory at Caltech, in which role his physical intuition and enthusiasm is by common consent acknowledged to have been a powerful stimulus for a generation of students and graduate students. His geniality is legendary.

Fowler's best-known contribution to the understanding of the origin of the elements was by means of his collaboration with Dr (now Sir) Fred Hoyle (now visiting professor at University College, Cardiff), Dr Geoffrey R. Burbidge (director of the Kitt Peak Observatory, Arizona) and Dr Margaret Burbidge (University of California, San Diego). The first complete statement of their solution, a monumental paper now generally known as "BBF&H", appeared in 1957 (*Rev. mod. Phys.* **29**, 547-650; 1957).

One of the obvious difficulties for the Nobel committees must have been that the prize by-laws prevent the splitting of a prize between more than three people. Another is that similar proposals to those in BBF&H were put forward at about the same time in a report by A.G.W. Cameron circulated by the Chalk River Laboratory of Atomic Energy of Canada Limited (CRL-41).

The distinctive feature of BBF&H is that it is, above all, a comprehensive paper. The synthesis of helium from hydrogen by the intervention of C, N and O nuclei as catalysts had been known from Bethe's work in 1938. Hoyle had argued in 1946 that the synthesis of nuclei of elements in the iron group could be accounted for by a kind of thermodynamic equilibrium at stellar temperatures, pointing to the potential importance of neutron addition. Most theories seeking to account for the occurrence of heavy elements, such as those of Teller and Gamow, had however supposed that the process responsible would have been a feature of the primordial unexpanded universe (although BBF&H acknowledges that theories due to Klein and, separately, Beskow and Treffenberg in 1947 had been compatible with nucleogenesis within stars, the central feature of the new proposal).

The commanding thesis of BBF&H is its convincing demonstration that nucleogenesis in stars is a sufficient explanation of observed nuclear abundances, with the important astrophysical consequence that a substantial number of existing stars must have been formed from material generated in stars whose evolution had been completed.

Technically, BBF&H was most successful in accounting for the formation of those isotopes heavier than ^{16}O which are not also constituted from alpha-particles

W.A. Fowler

by the addition of neutrons to lighter nuclei. Two distinct processes were suggested — the *s* (for *slow*) process, by means of which heavy isotopes newly formed by neutron addition usually decay (by beta-decay) before the addition of another neutron, and the *r* (for *rapid*) process in which the neutron flux would have been sufficiently great to have generated nuclei heavier than bismuth.

One of the essential steps in the argument of BBF&H was Fowler's confirmation, by means of experiments at Caltech, of an earlier prediction by Hoyle that there should be an excited state of the ^{12}C nucleus at an energy of about 7.6 MeV. Hoyle's prediction was derived from the assumption that the beginning of the red giant phase of the evolution of a star is marked by the onset of helium burning, in turn requiring a rate for the reaction between ^{8}Be and ^{4}He so great that it could be most simply accounted for by a near-coincidence ("resonance") of the combined energy of the two component nuclei with that of an excited state of ^{12}C.

The theory of nucleogenesis of BBF&H also stands out for the convincing identification that it first provided of realistic nuclear reactions that might serve as neutron sources in stars towards the end of the red giant phase. Fowler seems to have been chiefly responsible for drawing attention both to mechanisms by which odd-mass isotopes such as ^{21}Ne may be formed (by proton reactions) and then serve as neutron sources (by interaction with ^{4}He nuclei). It seems, however, to have been Hoyle who first recognized the usefulness of the data on neutron UN conference on the Peacefire Uses of Atomic Energy in August 1955. The Burbidges, among other things, were able to contribute data on their observations of stars containing heavy elements in their envelopes.

BBF&H is a landmark in the development of astrophysics for several reasons. The provision of a coherent explanation of nucleogenesis is one. Second, it settled once and for all an issue that had still be in doubt in the previous decade — the direction of stellar evolution along the Herzsprung-Russell diagram. Finally, it provided the objective basis for the calculations of the internal constitutions of stars that have since been carried out (crucial, for example, in the understanding of the place of globular clusters in the evolution of galaxies) as well as for the general understanding that the end-point of the evolution of a reasonably massive star is a supernova explosion.

The work described in BBF&H was begun at Cambridge (England) during Fowler's tenure of a Fulbright professorship in 1954–55. The original version of the paper was rejected by the first US journal to which it was sent, but snapped up by Dr Lewis Branscomb, then editor of *Reviews of Modern Physics*. The final version of the paper was worked through by Hoyle and Fowler in 1957 when both were attending a meeting of the Pontifical Academy of Sciences at the Vatican. **John Maddox**

0028-0836/83/430759-01$01.00

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Dwarf star instability

DR Subrahmanyan Chandrasekhar, who shares this year's physics prize with W.A. Fowler (see previous page) for his contributions to the understanding of stellar structure, was born in Lahore (now in Pakistan), educated at Madras and became a graduate student of R.H. Fowler at Cambridge, England, in 1930. His five years at Cambridge were embittered by a dispute with Eddington over the work now singled out by the Nobel committees.

Fowler had already argued that the behaviour of dwarf stars (with no substantial internal source of energy) should be influenced by Fermi statistics, the thermodynamic consequence of the Pauli exclusion principle which among other things ensure that the compressibility of condensed matter should be determined largely by electron exclusion. Chandrasekhar arrived in Cambridge with a solution of the non-relativistic (low-mass star) problem showing that the density of dense matter should vary as the 3/5ths power of the pressure and be independent of temperature. He extended this to the relativistic case in his PhD thesis in 1933, showing that the density should then vary as the pressure to the power ¾.

With or without the relativistic correction, the dominance of Fermi statistics for degenerate matter such as the material of dwarf stars implies that stellar radii decrease as stellar masses increase. In the relativistic case, Chandrasekhar's calculation showed that there must be an upper limit to the mass of a stable dwarf star calculated as 1.4 solar masses for dwarf stars made of hydrogen and as 1.2 solar masses for iron stars.

Subrahmanyan Chandrasekhar

The Chandrasekhar limit played an important part in the theory of nucleogenesis chiefly by providing a basis for the argument that in the late stages of the evolution of a massive star, the collapse of the degenerate core should result in the increased production of neutrons required for the *r*-process. Chandrasekhar's argument also provided from the outset a natural explanation of the small masses of then known dwarf stars (mostly a fraction of the Sun's mass).

For a reason not fully explained, Eddington took against this idea. Chandrasekhar has described (BBC Radio Three) how, "in a very shattering experience for me personally", Eddington had described his work as "obviously false" at a section meeting of the International Astronomical Union in Paris in 1934, and how he had been denied a chance to defend himself by the then secretary of the section on stellar constitution, W.H. Russell. When Chandrasekhar was offered a post at the University of Chicago in 1935, Eddington appears to have advised him to jump at the chance.

During nearly half a century in the United States, Chandrasekhar has earned an outstanding reputation for stamina. Stung by his experience at Cambridge, Chandrasekhar "stated my views as clearly as I could . . . in a book" (*An Introduction to the Study of Stellar Structure*, 1939) before turning his attention to the study of stellar opacity. In 1952, he became the managing editor of *Astrophysical Journal*, then published by the University of Chicago, and in that role served both as editor and single-handed referee for what the journal published.

In the early 1960s, Chandrasekhar converted himself into a relativist by means of a sabbatical spell at the California Institute of Technology. His monumental book *The Mathematical Theory of Black Holes* (Cambridge, 1982) includes one chapter nearly a hundred pages long to which a footnote apologetically explains that much of the mathematics has had to be omitted, for which reason a number of notebooks and more than 500 handwritten pages of algebra have been deposited with the library of the University of Chicago.

John Maddox

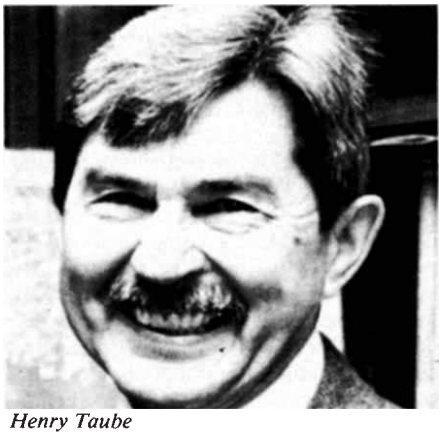
Electron transfer comprehended

THE Nobel Prize for chemistry was awarded to Dr Henry Taube of Stanford University in California for his fundamental studies of inorganic reaction mechanisms. Taube's most far-reaching work on electron transfer processes, cited in the Nobel award, was carried out 30 years ago. This work provided the foundation for an understanding of reaction rates and mechanisms in a wide range of oxidation-reduction reactions.

Taube's own work, however, has concentrated on often very simple inorganic systems, especially transition-metal complexes. From these elegant basic studies, Taube developed rules for predicting the rate of electron-transfer reactions as a function of electronic configuration, solvation and other factors. Colleagues credit him with almost single-handedly transforming inorganic chemistry from a descriptive to a predictive science.

Taube distinguished between two sorts of redox reactions in inorganic systems: "outer sphere" reactions involving the direct transfer of an electron between two molecules, as in the reaction between O_2 and O_2^- and "outer sphere" reactions involving the transfer of an atom, typically a shared ligand between two metals with coordination groups, as in the oxidation of Cr(II) to Cr(III) and the simultaneous reduction of Co(III) to Co(II) by transfer of a chloride atom between the metals.

Although Taube himself has never worked with biological systems, his results have had a profound effect on the understanding of enzyme kinetics. His theoretical work is invoked to explain why different enzyme reactions proceed at different rates and how rates change with changing conditions. Taube's rules also help to explain many chemical curiosities and many basic features of chemical behaviour that chemists have tended to take for granted (or have shrouded in mystery through the invocation of the magic word "catalysis"). One example: molecular oxygen, from a thermodynamic viewpoint, is a very powerful oxidizing agent; yet kinetically it is a reluctant one. Taube has shown that the addition of the first electron to O_2 is into an antibonding level which causes an unfavourable change in geometry, whence the kinetic barrier.



Henry Taube

Taube was the first to use isotopically-labelled oxygen in inorganic reaction studies and pioneered the application of nuclear magnetic resonance to the field.

Taube says that he made a conscious decision to concentrate on inorganic chemistry after teaching an advanced chemistry course and seeing the vast opportunities for research in what was — and, relatively speaking, still is — a wide open field. Taube is not well known outside chemistry, but he has for years received recognition from within the ranks of his discipline.

Stephen Budiansky

0028-0836/83/430760-01$01.00

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Medical genetics

Retinoblastoma and recessive alleles in tumorigenesis

from Fred Gilbert

USE OF a new method for detecting somatic genetic changes has yielded data giving strong support to the hypothesis that retinoblastoma — an intraocular tumour of children — results from the development of homozygosity for a mutant allele on chromosome 13. The data (see this issue, p.779) makes it possible to separate the different ways in which homozygosity might come about and shows that, in the cases examined, errors of mitosis or recombination events during mitosis, are often to blame[1]. Somatic inactivation of genes on chromosome 13 (through mutation, deletion, or rearrangement), hypothesized by Godbout *et al.* [2] in *Nature* a short while ago to be a common event in retinoblastoma, is clearly shown to be but one of several mechanisms by which the effective loss of activity of these genes may be achieved in this tumour.

All forms of retinoblastoma have been related to some kind of abnormality involving band q14 on chromosome 13. Retinoblastoma can occur in association with a constitutional deletion of the long arm of chromosome 13 that always includes band q14[3,4]. Deletions and rearrangements of chromosome 13 resulting in the loss of structural material from band q14, as well as monosomy for chromosome 13, have also been seen in direct preparations of retinoblastoma cells from individuals with normal constitutional karyotypes[5,6]. Similar abnormalities have been seen in cells from patients with both the sporadic and heritable forms of the tumour. All the evidence suggests that band q14 contains a gene that in some way contributes to tumorigenesis in retinoblastoma.

The available evidence can be best explained if retinoblast transformation requires (at least) two gene changes — the so called 'two-hit' hypothesis put forward by Knudson[7]. Retinoblastoma of both eyes is always associated with the heritable form of the disease while that of one eye is usually associated with the sporadic form. In heritable cases, it is argued, the first gene change is present in all cells, including germ cells, and can be transmitted in a dominant fashion; transformation requires a second gene change in a susceptible target cell, a retinoblast, already carrying the first 'hit'. In sporadic cases, both 'hits' presumably develop as chance events in the same retinoblast.

If only two gene changes are sufficient for tumorigenesis in retinoblastoma and one of those changes involves a locus at q14 on one chromosome 13 (ref. 8) then the second gene change could occur in the gene already carrying the first 'hit', in a completely different gene in the genome, or in the homologous locus on the other chromosome 13.

The first possibility can be ruled out as a general explanation for all retinoblastomas because of the existence of constitutional 13q deletion patients in whom the deletion represents the first 'hit'. The second possibility cannot be excluded at this time. Evidence in support of the third possibility — that retinoblast transformation may be the product of recessive alleles at 13q14 — has recently been presented by several laboratories[1,2,9,10].

These studies take advantage of the close linkage between the putative retinoblastoma locus (designated Rb-1) at 13q14 and the enzyme esterase D[10], and the development of a library of DNA fragments specific for 13q which reveal restriction fragment length polymorphisms (RFLP) along that chromosome[11]. The RFLP make it possible to distinguish between the members of a pair of chromosomes 13 in a cell line. And, as described by Cavenee and colleagues in this issue of *Nature*[1], the RFLP provide a means of determining which of several possible mechanisms underlies the expression of a recessive allele at a particular locus. In addition to deletion and point mutation, the mechanisms discussed (see their figure on p.780) include chromosomal non-disjunction with chromosome loss (non-disjunction is an occasional error of mitosis in which both chromatids of one chromosome go to the same pole at anaphase, leaving one daughter cell with only one member of a chromosome pair), non-disjunction with chromosome re-duplication (re-duplication results in two copies of the same chromosome in a cell), mitotic recombination, and gene conversion.

A patient has, for example, recently been reported with bilateral retinoblastoma and a normal constitutional karyotype, whose somatic cells contained 50 per cent of normal esterase D activity[9]. In previous cases, the only individuals with 50 per cent activity were those that carried a constitutional 13q deletion. Cells from the patient's tumour, however, contained no esterase D activity and only one grossly intact chromosome 13. Given the close linkage between esterase D and Rb-1 and the fact that all bilateral cases are heritable (hence, all of the patient's cells are presumed to carry the first 'hit', a deleted or mutated Rb-1), the simplest interpretation of these findings is that one of the chromosomes 13 in all of the patient's cell contains a sub-microscopic deletion spanning the esterase D locus and Rb-1. It is this chromosome that is presumably retained in the tumour cells.

Cavenee and colleagues[1] have carried work on this patient a stage further and used RFLP to analyse the tumour cells. They are able to demonstrate that a rearranged chromosome 13 is not likely be among the unidentified marker chromosomes in the tumour and that the loss of the 'non-deleted' chromosome 13 is a reasonable explanation for the observed lack of esterase D activity.

A second study, from Godbout and colleagues[2], reported on retinoblastomas from patients who were heterozygous for electrophoretic variants of esterase D. In four retinoblastoma cases (two unilateral, two bilateral), the tumour cells expressed only one of the two esterase D alleles. All of the tumours contained (at least) two apparently intact chromosomes 13 per cell. The authors discuss several possible explanations for the loss of esterase D activity in the absence of a visible 13q deletion or of monosomy for chromosome 13 in every cell; including somatic mutation, sub-microscopic deletions or chromosome rearrangements involving the esterase D locus, mosaicism for a 13q14 deletion, and non-disjunction with chromosome reduplication.

Mosaicism was dismissed as unlikely after it was shown that multiple fibroblast sub-clones from two patients whose tumours were homozygous for one esterase D allele expressed both forms of the enzyme. Non-disjunction with reduplication was considered unlikely because in one patient (RB412), both chromosomes 13 could be distinguished from one another: one contained fluorescent satellites on its short arms, the second did not. Both chromosomes 13 were present in every fibroblast and tumour cell analyzed.

The authors concluded that somatic inactivation was most likely to account for the loss of esterase D activity in these tumours and that somatic inactivation of genes near the esterase D locus, including Rb-1, was likely to be a prerequisite for tumorigenesis in retinoblastoma.

Now, Cavenee and colleagues, using recombinant DNA probes that identify RFLP at sites above and below the segment containing EsD and Rb-1 (between the centromere and 13q ter, respectively), have arrived at different conclusions to explain the inactivation of EsD (and the loss of the normal allele at Rb-1) in three of the tumours described in ref. 2. In the bilateral case RB409, for example, the patient's fibroblasts were heterozygous for RFLP expression at all three loci, while the tumour cells expressed only the paternal forms of all three RFLP. Since the tumour cells contained two chromosomes 13, this finding is consistent with the loss of the maternally-derived chromosome 13 and the re-duplication of the chromosome 13 contributed by the father. Presumably, the

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paternal chromosome 13 carries the germline first 'hit' at the Rb-1 locus, making the tumour cells homozygous for the mutant allele.

In RB412, the patient's fibroblasts were heterozygous for three RFLP, one mapped above and two below 13q14. The tumour cells contained the two structurally distinguishable chromosomes 13 seen in the somatic cells (with one marked by fluorescent satellites on its short arms) and continued to express both forms of the RFLP assigned to the segment between the centromere and 13q14. However, at the two RFLP mapped below 13q14, the forms contributed by only one of the parental chromosomes were expressed. This is consistent with a mitotic recombination event resulting from a break and cross-over at a point between the RFLP mapped above 13q14 and 13q14. Homozygosity for all loci distal to the break point would, then, be produced and would explain the loss of one form of EsD and one form of each of the two distal RFLPs; one Rb-1 locus would also be lost and presumably the locus retained was that carrying the first 'hit'.

In RB430, only one RFLP showed heterozygosity in the patient's fibroblasts and homozygosity in the tumour. As the RFLP in question has been mapped to the region containing EsD and Rb-1, homozygosity at all three loci could be explained by chromosome non-disjunction and re-duplication or by mitotic recombination; it is less likely to have occurred as the result of somatic inactivation (multiple point mutations. sub-microscopic deletions, and so on)[2].

In addition to providing evidence that homozygosity for a recessive allele at a locus on chromosome 13 may lead to the development of retinoblastoma, the approach described by Cavenee and colleagues utilizing RFLP, has broader implications for the study of somatic cell genetics. They have clearly demonstrated the value of chromosome-specific RFLP as a tool for the analysis of gene inactivation and its ability to identify the exchange of segments between chromosomes, below the limits of resolution of the light microscope. With the development of recombinant DNA probes for RFLP on chromosomes other than number 13, the approach they have outlined should also prove useful in studies of the expression of recessive alleles in other genetic systems and in the analysis of the roles played by single genes in tumorigenesis and tumour progression. □

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Biophysics

Muscle crossbridges seen by synchrotron light

from Maxine Clarke

FORCE generation in muscle is generally accepted to be the consequence of the cyclical action of crossbridges[1] which cause the thick filaments (composed of myosin) and the thin filaments (composed of actin) making up muscle fibres to slide past one another. Numerous techniques have been used to investigate the properties of muscle and the kinetics of the actomysin ATPase[2] (which catalyses the hydrolysis of ATP which powers muscle contraction) and the rapid mechanical properties of contracting muscle[3] have been characterized in some detail. Little direct information is, however, available about the supposed changes in conformation of attached crossbridges during energy transduction. One approach, the first detailed results from which appeared in a recent issue of the *Journal of Molecular Biology*[4], uses the very powerful X-rays produced by a synchrotron to provide extremely rapid X-ray diffraction patterns from contracting muscle.

One method of investigating crossbridge structure during the working stroke has been to isolate equilibrium states of the crossbridge cycle, on the assumption that such states are analagous to those that occur transiently during contraction[5]. Two equilibrium states are well understood: rigor (ATP free), in which nearly all the available crossbridges are attached to actin filaments; and relaxation, in which they are detached from actin. The latter state is produced experimentally by adding ATP, which causes crossbridges to detach, in the absence of Ca^{2+}, the ion which would allow the hydrolysis of ATP and the muscle power stroke to proceed. AMPPNP, a nonhydrolysable analogue of ATP, produces an equilibrium state quite distinct from relaxation or rigor[6], but in which mechanical measurements indicate that the same number of crossbridges are attached to actin as in rigor[7,8]. The structure of such AMPPNP crossbridges is not yet completely understood, but low-angle X-ray diffraction experiments on insect flight muscle have suggested that, in AMPPNP, the ends of the crossbridges nearest the thick-filament backbone retain the myosin periodicity and the ends nearest the thin filaments take up the actin periodicity[9]. The AMPPNP crossbridge may thus represent an alternative attached state which is similar to one of the transiently attached states occurring during contraction[10]. However, this is not the only way in which the AMPPNP data may be interpreted[5,11].

Low-angle X-ray diffraction patterns of muscle can in principle reveal the arrangement of the crossbridges with respect to the thick and thin filaments. Such measurements had previously shown little about the conformations of active crossbridges. New techniques utilising synchrotron radiation, combined with sophisticated X-ray detectors and data storage systems, are now allowing the intensities of the strongest reflections from a contracting muscle to be obtained in 1 ms (refs 4,12).

In the recent work[4] from Hugh Huxley and his colleagues, an isometrically contracting frog muscle has been quickly stretched or released by the approximate amount a crossbridge moves during its working stroke (100 Å) in an attempt to synchronize the displacement of crossbridges attached to actin and so reveal the resulting internal structural changes. The study mainly concerns the strong myosin-based 143 Å meridional reflection which has a high intensity during contraction and is considered here to arise from attached crossbridges. The intensity of this reflection decreases by up to 80 per cent after either a quick release or stretch of the muscle, and this intensity change can be reversed upon quickly readjusting to the original length. If the length readjustment is performed after 5 ms, by which time the 143 Å intensity has recovered, the intensity then falls again, which implies that the crossbridges have detached from and reattached to actin.

If an isometrically contracting muscle is allowed to shorten very slowly, the 143 Å intensity remains high. In this case one would expect there to be a large number of possible angles of crossbridge attachment to the slowly moving actin filament. In order to explain the high 143 Å intensity in these conditions, the suggestion is made that it originates from the inner (backbone)

0028-0836/83/430761-02$01.00

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end of the crossbridges, and that this region of the crossbridge remains well ordered with respect to the thick-filament periodicity during isometric contraction or slow shortening. After a quick length change, however, it is proposed that those crossbridges that are carried beyond the end of their working stroke remain rigidly attached to actin, causing a distortion of the myosin-based periodicity of the backbone end of the crossbridge and hence a drop in the 143 Å intensity.

The actin-based layer line pattern which is characteristic of rigor was not detected either during isometric contraction or after a rapid length change. The 143 Å reflection clearly does indicate, however, that changes in the crossbridges occur during contraction. There is still some controversy over how much of the 143 Å intensity arises from attached crossbridges, or indeed how many crossbridges are attached to actin filaments during normal contraction (for example, refs 5,12).

The data of Huxley and colleagues provide further evidence for the cyclical attachment and detachment of crossbridges to the actin filaments, where the crossbridge is considered as a single particle which produces force during the cycle by tilting about an extensible hinge region[13]. It remains to be seen how this view of the crossbridge cycle, in which the outer end of the bridge moves with the actin filament and the inner end stays associated with the thick filament, can be correlated with the other large body of independent data on crossbridge structure obtained from looking at equilibrium states. It will probably be necessary to have more information about the weaker reflections in the X-ray pattern from contracting muscle, particularly those with actin-based periodicity, in order to compare such crossbridge structures with, for example, the AMPPNP state. □

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Ecology

Animals as nutrient carriers

from Peter D. Moore

It has long been recognized that the movement of grazing animals from one terrestrial ecosystem to another, feeding in one and defaecating in the other, may result in a significant movement of certain elements between them. What has now been made evident, in work on the coral reefs of the Virgin Islands[1], is that a similar process takes place in aquatic ecosystems.

If the grazing and defaecation processes were random, there would be no net transfer of elements between ecosystems, but very often the behavioural patterns of herbivores result in an imbalance. Take, for example, roosting birds. Work on roosts of the rook (*Corvus frugilegus*) in England[2] showed that over 4 kg sodium, 8 kg potassium and 75 kg calcium were deposited per m^2 in faeces during an 8-week observation period. When compared with the average input from rainfall for an equivalent period, this represents twice as much sodium, 10 times the potassium and 35 times the calcium derived from rain. In addition there were further inputs from regurgitated grit.

Clearly such enrichment will affect the vegetation of the roost site; but will the ecosystems from which the nutrients are derived suffer some degree of impoverishment? No certain answer can be given. The lack of nutrients in many heavily sheep-grazed chalk grasslands has been blamed upon the non-random pattern of grazing and defaecation[3]. In the New Forest's acid grasslands large herbivores such as cattle, ponies and deer use specific latrine areas[4] which gain nutrients rapidly, but whether there is significant nutrient depletion elsewhere has yet to be demonstrated. The long-term harvesting of sheep from Pennine moorland may have been responsible for the low nutrient status of the upland pastures[5], but nutrient budget studies show the drain is negligible[6] in comparison with other gains and losses.

Turning to aquatic ecosystems, the utilization of lakes for the aquaculture of fish can result in nutrient imbalance. Balance sheets for the culture of rainbow trout in cages in Polish lakes[7] show the quantities of nutrients supplied to the fish in food exceed those recovered in the harvest of fish, the remainder entering the lake ecosystem in the form of waste food, dead (or escaped) fish and their faecal products. The recovery of elements such as phosphorus and nitrogen is only about 20 per cent of the input with the result that these elements are supplied to the lake at rates of about 0.6–1.0 g P and 2.7–4.4 g N $m^{-2}\,yr^{-1}$. This is clearly an important source of eutrophication in the lake.

Free-living fish can act as carriers of nutrients from one part of an aquatic ecosystem to another, as has recently been demonstrated by Meyer, Schultz and Helfman[1] in the coral reefs of the Virgin Islands. They have investigated the possibility that fish schools sheltering in coral reefs may enrich their environment with faecal and excretory products. Water samples were taken in coral heads where fish were resting and those in which they were not present and they found that levels of ammonium ions reached 0.9 μM in the former and only 0.2 μM in the latter. In addition, particulate matter rich in phosphorus and nitrogen was significantly more abundant when fish schools were present, presumably being derived from faecal material.

Both ammonium ions and the particulate matter form a readily available nitrogen source for the corals, but diffusion out of the vicinity, if rapid, could reduce these levels. Measurements of the diffusion rate, made by injecting dye into a coral head, showed that diffusion was, in fact, quite slow: it took over six minutes for the dye to be diluted to one-tenth its original concentration.

The total input of nitrogen by the fish is equivalent to 30–75 per cent of that derived from nitrogen fixation by blue-green algae. The question still remains, however, whether the additional nutrients permit higher growth rates among the corals. This was tested by marking selected coral heads and collecting the additional calcium carbonate deposited after a year had elapsed. Fish were then removed from certain heads and all corals were measurd again after a further eight months. In heads where fish schools had been removed, coral growth was only 55 per cent that expected, indicating that the presence of the fish is advantageous for maintained growth.

Mobile animals in aquatic ecosystems clearly play a part in the spatial redistribution of nutrients and, in doing so, influence the growth and performance of other organisms. In this respect they resemble their terrestrial equivalents. One added complication in aquatic habitats is the three dimensional nature of the mosaic produced by fish defaecation. □

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0028-0836/83/430762-01$01.00

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Medical genetics

Collagen and inherited connective tissue diseases

from Bryan Sykes

COLLAGEN aficionados have long cherished the idea that abnormalities in their protein lay behind the inherited disorders of connective tissue. This complex mixture of clinical entities presents with mechanical failure of tissues like bone, gut and blood vessel walls, ligaments, tendon and skin, often with disastrous results. Since collagen is the principal stress-bearing component of the susceptible tissues their belief is reasonable but, largely owing to the complexity of the molecule, substantiation has till now been anecdotal, meagre and indirect. Obviously, collagen structural genes are the prime candidates for the mutant loci and gene probes now being used to test the hypothesis are coming up with evidence to support it. It is too early to say whether the understanding of collagen gene mutation will ever reach the levels of sophistication seen with globin but an interdisciplinary meeting* on osteogenesis imperfecta, heard the first encouraging reports that, one day, it might.

Collagen molecules consist of three collagen α-chains wound round one another in a regular helix. At least seven distinct α-chains have been found (labelled α 1(I) through to α 1(V), α 2(I) and α 2(V)) and combine in different types of collagen molecules — types I and II, III being the major consitutents of connective tissues.

Fibroblasts cultured from a perinatal lethal case (Type II disease) synthesised an α1(I) dimer disulphide bonded in α_1 CB6 towards the carboxy terminus of the helical region (B. Steinman, Universitats-Kinderklinik, Zurich). This defect probably arose from a glycine-cysteine substitution and, since neither parent was affected nor synthesized the abnormal chain, a new dominant mutation was proposed. Any substitution for glycine in the helix would prevent perfect packing, though it was conceded during discussion. (A. Miller, University of Oxford) that the degree of distortion is unknown. To explain the severity of the mutation of a single allele it was suggested (B. Sykes, John Radcliffe Hospital, Oxford) that since each Type I molecule contained two α1(I) chains the gene product of one mutant allele could theoretically be incorporated into three-quarters of the molecules. Taken together with the drastic effects of glycine substitution on helix stability this may explain the high mutation rate in many collagen diseases.

Studies on cultured fibroblasts (A. Nicholls, MRC Clinical Research Centre, London) had also led to the discovery of a patient who failed to incorporate pro-α2(I) precursor chains into mature Type 1 molecules. The symptoms were surprisingly mild, presumably because compensatory formation of $[\alpha 1(I)]_3$ trimer was an effective mechanical substitute. Though pro- α2(I) levels were normal, S_1 mapping revealed that the patient was homozygous for a mutation at the 3′ end of the gene corresponding to the C-propeptide region, the effect of which was to block incorporation (T. Pihlajamiena, Rutgers Medical School, New Jersey). The parents were consanguinious and heterozygosity was confirmed.

Much more perplexing was a 300 base pair deletion at the 3′ end of the enigmatic α1(I) like gene found in four out of six perinatal lethal cases from widely different ethnic backgrounds (F.M. Pope, MRC Clinical Research Centre, London). This gene, localised to chromosome 12, shares sequence homology with α1(I) (chromosome 17) but is clearly distinct from it. That it may be important in cartilage or bone formation was suggested by its strong hybridization to mRNA from a rat chondrosarcome (K.S.E. Cheah, Imperial Cancer Research Fund Laboratories, London). However, one parent in each case was heterozygous for the deletion also and one parent at least had, in retrospect, symptoms of other connective tissue disease. Irrelevant normal variants of the gene were unlikely explanations since about 400 other individuals did not have a deleted allele and the consensus was a possible compound heterozygosity.

The same gene was used to illustrate testing for causal mutations by segregation of single and multiple restriction site variants (B. Sykes) who showed how it could be excluded from causality in two pedigrees of dominantly inherited Type 1 disease.

Most delegates left the meeting optimistic that the disease loci have been identified and that the effort of many laboratories will one day be rewarded by a unified theory whereby collagen structural gene mutations will explain first the biochemistry, then the complex clinical and genetic picture of these crippling diseases. □

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The Second International Symposium on Osteogenesis imperfecta: Brittle Bone Syndrome was held in Oxford, August 30-31, 1983.

Astronomy

Structure and evolution of the Magellanic Clouds

from Sidney van den Bergh and Klaas S. de Boer

RECENTLY over a hundred astronomers from the northern and southern hemispheres gathered to discuss the interpretation of new observations of the Magellanic Clouds*. Since the last such meeting, in 1969, understanding of the Magellanic Clouds has been revolutionized by the completion of 4-m class telescopes in the southern hemisphere (Cerro Telolo Inter-American University and European Southern Observatory telescopes in Chile, and the Anglo-Australian telescope in Australia), by the development of highly efficient panoramic detectors such as the charge coupled device, and by observations from space.

Because of their proximity the Large and Small Magellanic Cloud are crucial stepping stones between our own Milky Way system and the realm of distant galaxies. In particular the Clouds are the only external galaxies in which it is possible to study both the integrated properties of star clusters and the spectra and colours of individual cluster stars. Recent multicolour observations by L. Searle (Mt. Wilson Observatory, Pasadena) are able to order Cloud clusters by relative age while colour-magnitude diagrams of stars within some of these clusters provide an absolute age calibration. Such work has revealed striking differences between the evolutionary histories of the Large Magellanic Cloud and Small Magellanic Cloud on the one hand and the Galaxy on the other.

Exceedingly old clusters, similar to galactic globular clusters, are found to be relatively rare indicating that star and cluster formation got off to a rather slow start in the Magellanic Clouds, contrasting with our Galaxy in which an enormous burst of star and cluster formation seems to have taken place as the Milky Way system collapsed from its gaseous proto-galactic state. In the Galaxy this initial burst of star formation resulted in the subsequent production of vast numbers of supernovae, which rapidly built up the heavy element abundance in the galactic interstellar gas. The Magellanic Clouds seem to have collapsed more slowly; probably because

*IAU Symposium No. 108 on Structure and Evolution of the Magellanic Clouds was held in Tübingen, W. Germany 5-8 September, 1983.

0028-0836/83/430763-02$01.00

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their masses are much smaller than that of the Galaxy. As a result, the heavy element abundance in the interstellar gas of the Magellanic Clouds was enriched rather slowly so that the metal abundance in the Clouds was, at all times (J. Lequeux, Marseille Observatory), lower than that prevailing in the Galaxy during the same epoch. Presumably because of its lower mass, stars and gas in the Small Cloud are observed to have an even lower heavy element abundance than do the stars and gas in the Large Cloud.

R.J. Dufour (Rice University, Houston) showed that the products of primary nucleosynthesis, such as O, Ne and Ar, are depleted by a factor of ~ 6 in the Small Cloud and ~ 3 in the Large Cloud compared to their abundances near the Sun. Since interstellar grains are mainly composed of heavy elements, dust is much less ubiquitous in the Clouds than it is in the Galaxy; dust-to-gas ratios are ~ 4 and ~ 17 times lower in the Large and Small Clouds respectively, than they are in the solar neighbourhood (J. Kornneef, Space Telescope Science Unit, Baltimore). Since interstellar clouds are cooled by dust, the abundance of molecules (which form on dust in cold clouds) in both the Large and Small Clouds is also found to be much lower (F.P. Israel, European Space Agency, Noordwijk) than it is in the Galaxy. The fact that the Large Magellanic Cloud is somewhat dustier than the Small Magellanic Cloud may account for the fact that the hydrogen gas in the Large Cloud exhibits a much clumpier structure than does the almost dustless gas in the Small Cloud (S. van den Bergh, Dominion Astrophysical Observatory, Victoria). Such dustless gas will not cool and clump as much as the gas in dustier systems like the Galaxy and the Large Magellanic Cloud. It is rather ironic that star formation seems to be taking place so efficiently in the Small Magellanic Cloud even though it contains no giant molecular clouds and does not suffer spiral density wave shocks — the two factors which are generally considered to dominate star formation in the Galaxy.

J. Hutching (Dominion Astrophysical Observatory, Victoria) suggested that the relatively high frequency of black holes in the Clouds (two probable cases known in the Large Magellanic Clouds versus only one in the Galaxy) might also be due to the lower heavy element abundance. The stellar winds that cause mass loss from evolving stars in the Clouds will therefore be weaker than they are for similar stars in the Galaxy. As a result stars will die with higher masses and will be more likely to end their lives as black holes rather than as neutron stars. Low gas opacity (resulting from a low metal abundance) will also make infall of gas into the degenerate components of binary stars more efficient and lead to higher X-ray luminosities. This may explain why the X-ray sources in the Clouds are systematically brighter than their galactic counterparts.

Infra-red observations of cepheids in the Large and Small Clouds (M. Feast, South Africa Astronomical Observatory) and by D.L. Welch and B.F. Madore (David Dunlap Observatory, Toronto) have produced conflicting answers to the question: Is the slope of the cepheid period-luminosity relation dependent on metallicity? If the answer to this question should turn out to be affirmative it would make it more difficult to use cepheid variables to calibrate the extragalactic distance scale.

Considerable controversy still surrounds the question whether the object R136a in the Tarantula Nebula is a single superstar with a mass of some thousands of solar masses or if it is a very compact duster of stars with individual masses of the order of one hundred solar masses or less. It is hoped that new observations that are currently being planned will resolve this problem within the next few months. □

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Evolutionary biology

Sexual selection in hermaphroditic plants

from Andrew G. Stephenson

DARWIN put forward the theory of sexual selection to try to account for the evolution of traits that appear to have no direct value in the 'struggle for survival' but that allow an individual to have greater success in obtaining mates. Two processes were seen as important: competition among members of one sex for reproductive access to the other sex; and preference by members of one sex for certain members of the other sex[1]. Because female gametes are larger (and more 'costly') than male gametes, and because females generally invest more resources per offspring than males, males will typically compete for females[2] while females will typically exercise choice over their mates[3,4].

A few early investigators mentioned that sexual selection might occur in plants but only recently have theoreticians seriously explored the necessary conditions and potential consequences of sexual selection in hermaphroditic plants[4,5]. Now, theoretical work has been complemented by the appearance in a recent issue of *Nature* [6] of one of the first empirical studies of the role of sexual selection in the evolution of reproductive traits in plants.

According to the theorists [3,4] sexual selection should also be a potent force in the evolution of reproductive traits in outcrossing plants if resources (rather than pollination) regularly limit fruit and seed production. When these conditions are met, plants should evolve traits that allow them to compete for access to ovules of conspecifics and to influence which pollen grains sire their seed crops.

In the new work that has just been reported in *Nature* David Queller reports that the floral display of a hermaphroditic milkweed, *Asclepias exaltata*, has evolved through the male competition component of sexual selection[6]. The milkweeds are typical of most outcrossing hermaphroditic plants (including many of the most important economic species) in that they regularly produce far more flowers than they do mature fruits [7].

An unusual characteristic of the milkweeds made it possible for Queller to make quantitative comparisons of pollen donation, pollen receipt, and fruit production on experimentally manipulated inflorescences of *A. exaltata*. Milkweed flowers disseminate their pollen in packages called pollinaria (there are five pollinaria per flower). These pollinaria resemble minute saddlebags and attach to the legs or other appendages of insects visiting the flowers. Transport and insertion of only one pollinaria into the female portion of another flower is sufficient to initiate a fruit. It was therefore easy for Queller to determine the number of pollinaria removed by the floral visitors by examining the spent flowers with a dissecting microscope; and he could estimate the number of pollinated flowers merely by counting the number of fruits that begin development.

When Queller removed half of the flowers from inflorescences of *A. exultata* (to simulate the effect of producing fewer flowers) and when he excluded floral visitors from inflorescences for half of the 7–9 day blooming period (to simulate decreased flower longevity), he obtained some most interesting data. Compared to controls, fewer pollinaria were removed and fewer fruits were initiated. But the number of mature fruits produced by the experimental inflorescences did not decrease. As a full complement of fruits are produced on the experimental inflorescences despite a decrease in the number of pollinated flowers, Queller concludes, quite reasonably, that pollination does not limit fruit production. Furthermore, as the number of pollinaria removed is reduced on the experimental inflorescences, Queller suggests that floral number and longevity can be viewed as traits that increase the probability that a plant will donate pollen and, ultimately, produce offspring through the less costly male function.

0028-0836/83/430764-02$01.00

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Although the results of Queller's study are consistent with predictions generated by the theory of sexual selection, the study was conducted on only a single population during a single year. Because the frequency of pollinator visitation may fluctuate by year and location, the possibility that pollination commonly limits fruit production cannot be ruled out. In addition, little is known about the actual relationship between the number of pollinaria removed and the number of offspring sired.

If we set aside these potential problems, there is indeed a difference between the morphological and the functional sex of the flowers on *A. exultata*. While all of the flowers are bisexual morphologically (that is, they have both male and female parts), only a small percentage of those flowers are bisexual functionally (that is, they disseminate pollen and produce mature seed). It may, however, be premature to assume that the 'extra' flowers perform no female function. Janzen[7] and Charnov[4] suggest that the extra flowers on outcrossing hermaphrodites not only donate pollen but also collect pollen in order to provide a plant with a choice of which fruits to mature. Their suggestion is supported by two recent studies of the low-fruiting hermaphrodites, *Campsis radicans* and *Asclepias speciosa* which show these species selectively (non-randomly) mature the fruits sired by certain pollen donors. In addition, the preferred donors produce fruits that contain more and heavier seeds[9,10] and produce seedlings that are more vigorous[10] than those of the less preferred donors.

These studies raise the possibility that the advantages of female choice may equal or exceed the advantages of producing extra flowers for their effects on male competition. Queller, however, presents phenological data for *A. exaltata* that support male competition as the primary reason for the extra flowers. Unfortunately, these data are circumstantial. A more direct, but far more difficult, approach would be to examine the variance in the number of offspring produced through the male and female functions in a population of hermaphroditic plants. By analogy to animal models[11,12], male competition should be intense if the variance in the male function exceeds that of the female function. □

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European Space Agency

Giotto spacecraft in danger?

from G.M. Coupé, R.M. Dean and R. Lainé

IN A recent *News and Views* article entitled 'Giotto spacecraft in danger' (*Nature* **303**, 659; 1983), M. K. Wallis, after some introductory remarks describing the Giotto mission in general, criticizes our analysis of the effect of impacting dust particles on the spacecraft (*ESA J.* **7**, 1, 15; 1983) on essentially two points. His conclusions differ from ours and he sees a "scientific controversy" which "needs to be resolved quickly". We are of the opinion that Wallis's criticism is unfounded.

First, Wallis claims that we have not included a momentum multiplication factor for the non-penetrating dust particles and refers to work by J.A.M. McDonnell of the University of Kent who has pointed out the need for such factors. In fact, in the chapter 'quantitative results' of our paper we explicitly state that we have included momentum multiplication, the factors being taken from a note of J.A.M. McDonnell (our ref. 7).

Wallis writes "Another feature of Giotto is that its shield and exposed instruments present asymmetric surfaces to the oncoming dust. The ESA scientists decided that because the spacecraft rotates, the net effect of unbalanced impacts would be zero". He then arrives at the conclusion that the mission appears at risk "not only from possible milligramme-sized impacts but also from the rain of non-penetrating microgramme dust", and proposes to attach "counterbalancing sections to the shield to improve symmetry". The third section of our paper actually discusses in detail the mathematics for a non-circular dust shield, and the equations clearly show that the body-referenced components of the nutation angle have non-zero mean values when dust shield extensions are present. It is also stated that the inertial components of the angular momentum vector drift have zero mean values due to the spacecraft rotation. Therefore, the total spacecraft spin axis perturbation has a non-zero mean value.

The spacecraft attitude perturbation must, however, be represented by both the mean values for body-referenced components of the nutation angle and inertial components of the angular momentum vector drift and by the corresponding standard deviations due to the random dust particle impacts. The standard deviations have been found to be far more significant, and the 50 per cent and 95 per cent confidence levels of the angular deviations presented in our paper are almost entirely determined by these standard deviations, whereas the mean value are negligible. It is useful to note that including the dust shield extensions caused the confidence levels to increase by 10.5 per cent, and adding counterbalancing sections as proposed by Wallis would make the situation even worse.

One other point in Wallis's note should be clarified. Wallis claims that we "devised a Monte-Carlo calculation to simulate stochastic impacts". Our results have been obtained fully analytically using the theory of stochastic processes (generalized Poisson step process). A Monte-Carlo simulation was only used to relate confidence levels of a two-dimensional gaussian distribution to its mean values and standard deviations.

As a concluding remark we consider the Giotto spacecraft attitude-perturbation behaviour as well understood, since it has been the subject of intensive study within the Agency since project approval. Our referenced article represents only a summary at one point in an area that is still advancing. □

At the 6th Giotto Science Working Team meeting, held at the European Space Agency project technical management centre (ESTEC) in Noordwijk, The Netherlands on 7 September 1983, the following joint statement was issued by G.M. Coupé and M.K. Wallis:

WE have discussed our differing opinions on the results of the ESTEC work on Giotto attitude perturbations during the Halley encounter due to the dust particle impacts.

The ESTEC results as based on the Divine JPL model appear correct within a couple of per cent. (A slight modification in the order of a few per cent could come from the non-zero averaging of the mean perturbation in inertial coordinates.)

ESTEC has taken the McDonnell multiplication factors into account as given in the 4th SWT meeting (factors 40 and 2), and did not observe any influence. M.K. Wallis points out that different multiplication functions need to be taken into account for parts of dust shield surfaces with different thicknesses.

The approach M.K. Wallis is working out is of a deterministic nature. It is expected to represent the mean attitude perturbation of the spacecraft during the fly-by, which will be appropriate in case the large particles would not be present in the Divine model. In addition the probability level of this mean attitude perturbation should be established. □

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0028-0836/83/430765-01$01.00

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Isotopically anomalous nitrogen in primitive meteorites

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The extreme enrichments in ^{14}N (up to 48%) found in acid resistant residues of the Allende and Murchison meteorites cannot be attained by normal Solar System processes and must therefore be due to stellar nucleosynthesis. Consequently, the xenon component enriched in the heavy isotopes associated with the light nitrogen very probably was made by stellar nucleosynthesis rather than by fission of an extinct superheavy element.

THE most ancient materials currently accessible to man are traces of presolar matter that have survived in primitive meteorites. They can be recognized by their anomalous isotopic compositions, lying outside the range for known or plausible Solar System processes[1,2]. We have recently found[3] several types of "heavy carbon" with $^{12}C/^{13}C$ ranging down to 42 ($\delta^{13}C = +1{,}100‰$) compared with the normal terrestrial ratio of 89. Herein, where δ values are used

$$\delta = ((R_{sample} - R_{standard})/R_{standard}) \times 1{,}000$$

where $R = {}^{13}C/{}^{12}C$ or $^{15}N/^{14}N$; standards are C, PDB and N, air. We have now measured nitrogen in some of the same samples, and have again found large isotopic anomalies: $^{14}N/^{15}N$ up to 406, compared to the Earth's atmospheric value of 272.

The analytical techniques used for nitrogen and other volatile elements (C, noble gases) are stepped pyrolysis or combustion; samples are heated for fixed intervals at increasing temperatures (in vacuum or in oxygen respectively) and the gas evolved is analysed in a mass spectrometer after purification[4]. Nitrogen from different components or sites is therefore released at various temperatures depending upon either the rate of diffusive loss (as in stepped pyrolysis) or the combustibility of the material (as in stepped combustion).

Since the very first measurements of nitrogen isotopic compositions in meteorites, it has been obvious that components of widely different $\delta^{15}N$ exist in carbonaceous chondrites. A very heavy component, of $\delta^{15}N = +170$ to $+190‰$, occurs in the unique C2R chondrite Renazzo[5–8] and in Y790003, an Antarctic C2M meteorite from the Yamato Mountains[9]. At the opposite end of the scale, a $\delta^{15}N$ of $-90‰$ has been measured for a 800–900 °C stepped heating fraction from the C3V chondrite Allende[10]. Stepped combustion[11] of Allende matrix also allowed recognition of an isotopically light component, $\delta^{15}N = -54‰$ for the 683–700 °C step. An early, indirect hint for a small, very light nitrogen component of $\delta^{15}N$ between -574 and $-1{,}000‰$ was inferred by Kung and Clayton[5] for an HCl/HF resistant residue from Allende from mass and isotopic balance for two different nitrogen analyses by pyrolysis and Kjeldahl dissolution. However, stepped combustion of an H_2O_2 treated HF/HCl resistant residue revealed no $\delta^{15}N$ less than $-115‰$[12].

As in the carbon isotope study[3], we have investigated mainly carbonaceous residues separated by chemical resistance and grain size and previously analysed for noble gases[13–15] (Table 1). At least three exotic (that is, that cannot be explained by normal Solar System processes) noble gas components have been recognized in varying proportions: (1) Carbonaceous chondrite fission (CCF) xenon, enriched in the heavy and light isotopes ($^{136}Xe/^{132}Xe \approx 0.64$, about twice the atmospheric value) associated with carbon of $\delta^{13}C \approx -38‰$. (2) s-process xenon, enriched in even numbered, middle isotopes ($^{130}Xe/^{132}Xe \approx 0.48$, about three times the atmospheric value) associated with $\delta^{13}C$ of $\sim +1{,}100‰$. (3) Neon-E(L), essentially monoisotopic ^{22}Ne ($^{20}Ne/^{22}Ne < 0.01$, compared with the atmospheric ratio of 9.8) with carbon of $\delta^{13}C$ about $+300‰$[16]. The impetus for the present work was the expectation that samples with anomalous carbon and noble gases would also contain unusual nitrogen.

All nitrogen released was quantitated and isotopically measured by a static mass spectrometer[4,15,17]. Although use of a static mass spectrometer for $\delta^{15}N$ measurements affords far greater sensitivity, the analysis of samples much smaller than those previously studied is not without problems. First, in some early experiments (Allende and Murchison bulk samples), the total sample intended for analysis did not transfer to the extraction vessel because of electrostatic problems. Second, in all cases of stepped combustion, there was incomplete recovery of released nitrogen for mass spectrometry. However, where comparisons are possible, the isotopic data agree with the literature values, suggesting that no isotopic fractionation occurred. Third, by virtue of its small sample requirements, the static mass spectrometer exaggerates problems of sample heterogeneity. For the above reasons, absolute yields reported are preliminary;

Table 1 Meteorite samples

Sample	Treatment	Mass(%)	C(%)	Xe^{132}	Xe^{136}/Xe^{132}	Xe^{136} (CCF %)	$\delta^{13}C$ (‰)
Allende							
Bulk	—	≡100	≡100	≡100	0.338	≡100	−17.3
BB	HF/HCl	0.253	87	90	0.340	97	−18.9
B1B	HNO_3, $Cr_2O_7^{2-}$	0.039	8	6	0.644	70	−30.4
CC	HNO_3, $HClO_4$	0.018					
Murchison							
Bulk	—	≡100	≡100	≡100	0.320	≡100	−10.6
2C10 f (<1 μm)	HF/HCl, HNO_3,	0.078	3.2	3.5	0.409	25	−26.1
2C10 m (1–3 μm)	NaOCl, $NaOH/H_2O_2$	0.048	0.11	0.0098	0.348	0.027	+11.8

Absolute values for the bulk meteorites are: C = 0.23 and 1.56% respectively; $Xe^{132} = 17 \times 10^{-10}$ and 95×10^{-10} cc STP g^{-1} respectively; $Xe^{136}_{CCF} = 0.48 \times 10^{-10}$ and 1.30×10^{-10} cc STP g^{-1} respectively.

0028–0836/83/430767—05$01.00

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they are probably low rather than high if the sample is not grossly heterogeneous. Relative yields from a single sample and isotopic compositions are more reliable.

Allende

Data obtained from a stepped pyrolysis of bulk Allende are shown in Fig. 1*a*. Both the total nitrogen yield and the cumulative isotopic measurements (17 p.p.m. and −36 ‰ respectively) are in the range of other published work: 18 p.p.m., −43 ‰ for pyrolysis of a bulk sample[5]; 21 p.p.m., −26 ‰ and 20 p.p.m., −34 ‰ for pyrolysis of coarse chips and fine powder respectively[10]; and 42 p.p.m., −29 ‰ for combustion of matrix material[11]. Some of the discrepancies between the different studies are probably due to the rejection of data below an arbitrary temperature (in our case, 300 °C) on the assumption that they represent adsorbed atmospheric nitrogen. Similarly, the proportion of matrix in the sample affects the results as much of the nitrogen is located in that part of the meteorite. It has already been established that an isotopically light nitrogen phase exists in Allende[5,10–12,15] and indeed a glimpse of it is seen in the 900 to 1,100 °C region of the stepped pyrolysis extraction (Fig. 1*a*). The rest of the nitrogen from Allende is rather ordinary, with $\delta^{15}N$ values between −15 and −20 ‰ except the 300 °C step which is probably atmospheric.

To investigate further the isotopically light nitrogen, we have analysed a suite of acid residues from Allende, each progressively more harshly oxidized. Nitrogen isotope data, acquired by stepped pyrolysis and combustion, for Allende BB are shown in Fig. 1*b*, *c*. The HF/HCl treatment, which destroys 99.7% of the meteorite, leaves 34% of the nitrogen originally present. Some of the isotopically heavy, low temperature nitrogen found in the bulk sample has been lost but the release temperature of the lightest nitrogen observed has not changed. The $\delta^{15}N$ for the nitrogen evolved between 900 and 1,200 °C is consistently lighter, suggesting that acid processing also eliminated some heavier material that was either protected within minerals or simply thermostable. It can also be seen from Figs 1*b*, *c* that, in the case of Allende BB, stepped combustion affords a lower resolution than pyrolysis for the various nitrogen species, as most of the gas was evolved in only three fractions, of which the middle one alone accounted for 76% of the total. Nitrogen released at high temperature by pyrolysis is liberated ~500 °C earlier in the combustion temperature program. This observation, however, could indicate that nitrogen is present in a combustible, presumably carbonaceous, host.

Nitrogen isotopic data acquired by stepped combustion for Allende BB and two further samples, B1B and CC (prepared from BB by destroying 80–90% of this by wet chemical oxidation), are plotted in cumulative release fashion in Fig. 2. All of the samples contain more than one nitrogen-bearing phase, but the lightest nitrogen observed monotonically progresses to a minimum $\delta^{15}N$ value of −326 ‰ for the 500–600 °C extraction of CC. (A replicate analysis on an 8 μg aliquot yielded a $\delta^{15}N$ value of −331 ‰, albeit with a larger uncertainity).

Allende CC has a substantial (23%) heavy nitrogen component, released at 700 °C. No such nitrogen appears in B1B; the 700 and 1,200 °C fractions account for only 0.2% of the total N. Presumably, the heavy component resists $HClO_4$ but not $Cr_2O_7^{2-}$ in our experimental conditions and hence was lost from B1B. This heavy nitrogen may actually consist of a small, very heavy, component swamped by a large amount of the preceding, light nitrogen of $\delta^{15}N = -326$ ‰. More elaborate chemical separations or a different temperature programme will be required to characterize such a minor component. Heavy nitrogen may be associated with s-Xe, Ne-E(L)[12] and heavy carbon[3] as all of these have been seen in high temperature extractions of Allende.

Murchison

The bulk nitrogen isotopic composition of Murchison is well established at somewhere between +39 and +44 ‰[5,7,15]. A stepped pyrolysis extraction (Fig. 3*a*) shows the existence of a

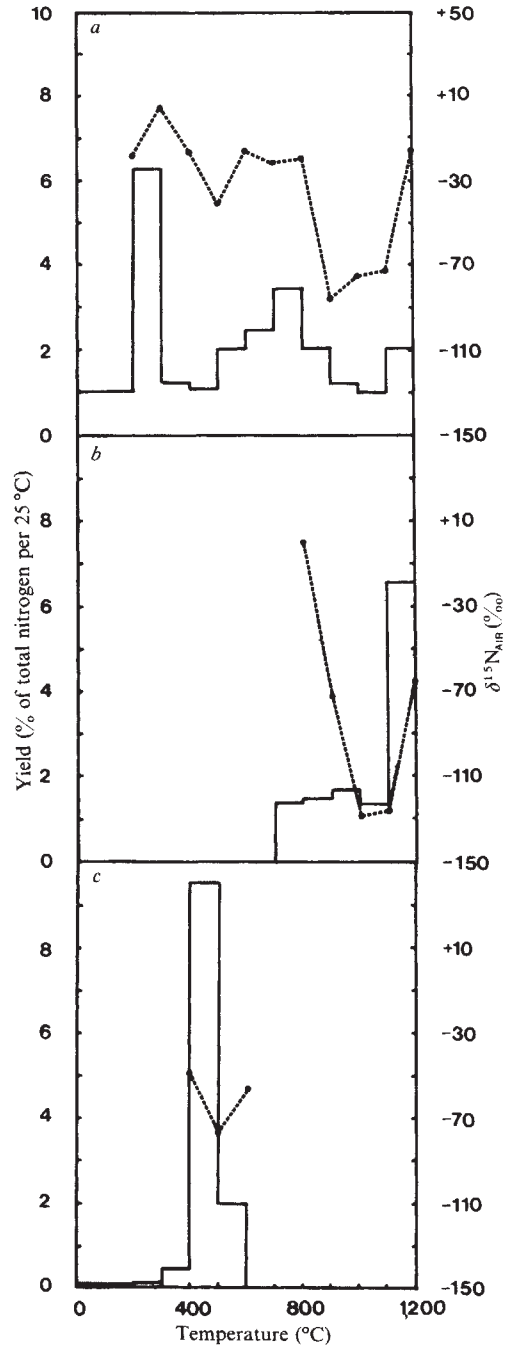

Fig. 1 Nitrogen release and isotopic composition from fractions of the Allende meteorite plotted against temperature of extraction. *a*, Stepped pyrolysis of a <50 μm sieved fraction, ΣN(300–1,200 °C) = 17 p.p.m., $\Sigma\delta^{15}N_{AIR} = -36$‰. *b*, Stepped pyrolysis of BB (see text) ΣN = 2,317 p.p.m., $\Sigma\delta^{15}N_{AIR} = -74$‰. *c*, Stepped combustion of BB ΣN = 906 p.p.m., $\Sigma\delta^{15}N_{AIR} = -73$‰

multicomponent mixture, the most significant $\delta^{15}N$ compositions being +41 ‰ (600–700 °C), +24 ‰(800–1,000 °C) and +135‰(1,200 °C). When the sample is subjected to stepped combustion, 80% of the nitrogen is released below 600 °C (Fig. 3*b*), again indicating a close association with a combustible, presumably carbonaceous, material. Some of the resolution afforded by the stepped pyrolysis has been lost because carbonaceous matter degrades over a narrower temperature range during combustion. Nevertheless, it is still just possible to distinguish two major releases, one from 200 to 600 °C and the other containing isotopically heavier nitrogen between 700 and 900 °C. The total nitrogen yields from both samples are low by comparison to previously published values, probably for the reasons discussed earlier, although it should be pointed out

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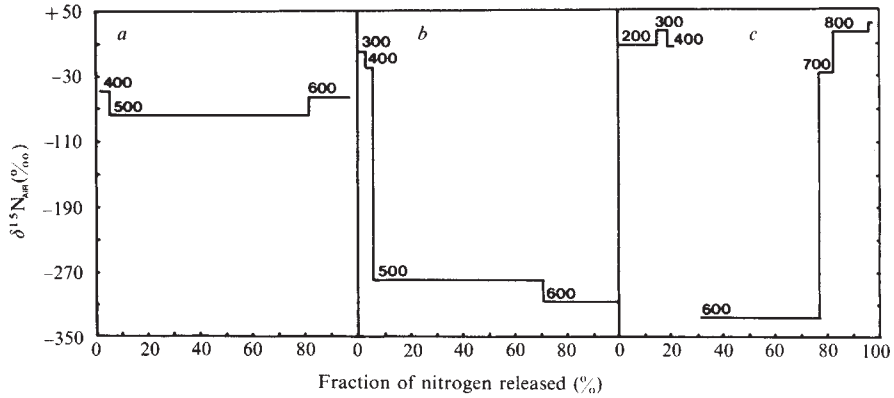

Fig. 2 Cumulative release plot of nitrogen liberated by stepped combustion of a series of progressively more harshly treated Allende acid resistant residues. The figures on the plots correspond to extraction temperatures. *a*, BB $\Sigma N = 906$ p.p.m., $\Sigma\delta^{15}N_{AIR} = -73$‰; *b*, B1B $\Sigma N = 904$ p.p.m. $\Sigma\delta^{15}N_{AIR} = -271$‰; *c*, CC $\Sigma N = 1{,}108$ p.p.m., $\Sigma\delta^{15}N_{AIR} = -164$‰ (not counting missing 500° fraction).

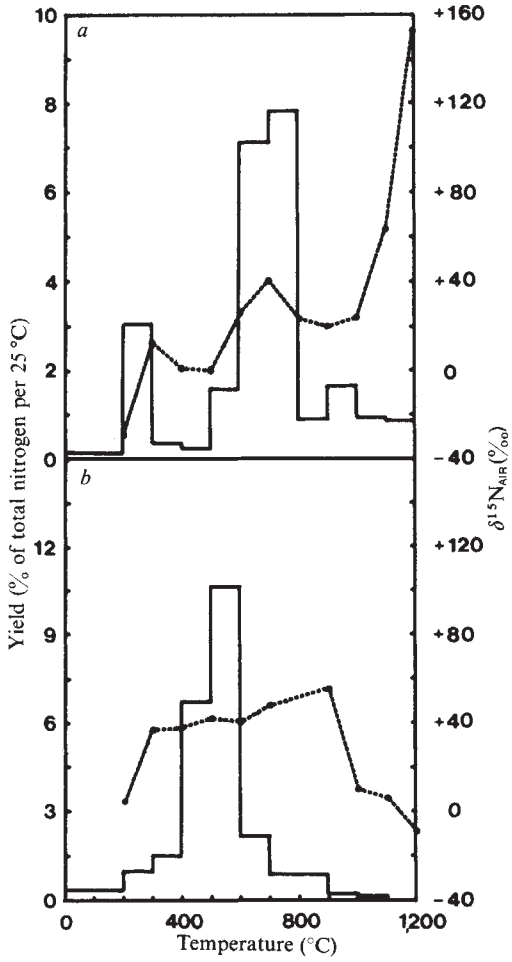

Fig. 3 Nitrogen release and isotopic composition from the bulk Murchison meteorite plotted against temperature of extraction. *a*, By stepped pyrolysis $\Sigma N(300\text{–}1{,}200\ °C) = 367$ p.p.m., $\Sigma\delta^{15}N_{AIR} = +36$‰; *b*, by stepped combustion $\Sigma N = 345$ p.p.m., $\Sigma\delta^{15}N_{AIR} = +43$‰.

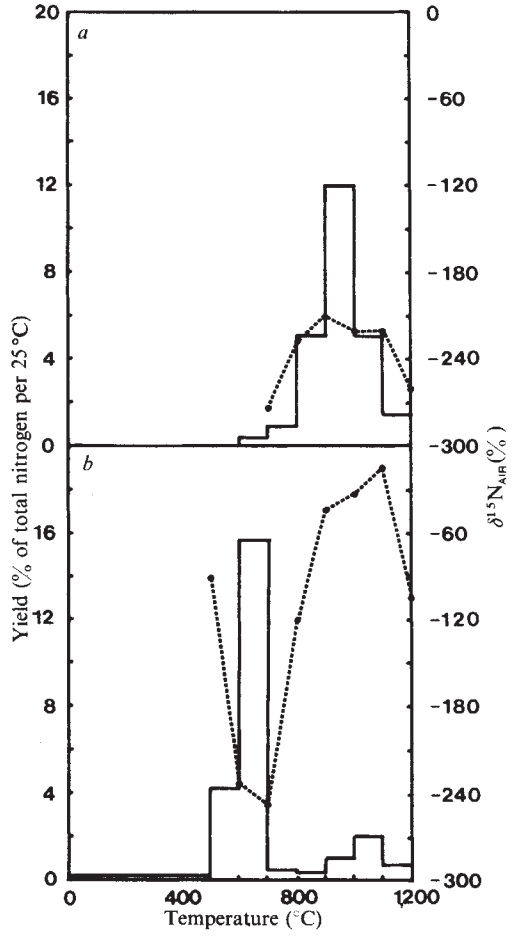

Fig. 4 Nitrogen release and isotopic composition from a <1 μm acid resistant fraction (2C10f) of the Murchison meteorite plotted against extraction temperature. *a*, By stepped pyrolysis $\Sigma N = 5{,}188$ p.p.m., $\Sigma\delta^{15}N_{AIR} = -222$‰; *b*, by stepped combustion $\Sigma N = 2{,}900$ p.p.m., $\Sigma\delta^{15}N_{AIR} = -205$‰.

that the sample of Murchison used in these experiments also had less carbon (1.5 wt %) than other specimens (2.0 to 2.5 wt %)[19–21].

Analyses of acid resistant residues that have been oxidatively processed to remove polymeric organics afford some insight into the nature of the nitrogen species. One such sample (Murchison 2C10f), a fine-grained (<1 μm) residue of the parent meteorite after treatment with HCl, HF, HNO_3 and NaOCl (ref. 13), has been analysed by both stepped pyrolysis and combustion (Fig. 4). Both techniques show conclusively that the bulk of the nitrogen is isotopically very light, having a $\delta^{15}N$ of between −210 and −274‰. In the pyrolysis experiment (Fig. 4*a*), the major temperature fractions have slightly heavier nitrogen than those for combustion ($\delta^{15}N \approx -220$‰ compared with −240‰) (Fig. 4*b*). Stepped combustion (Fig. 4*b*) reveals heavy nitrogen (~20% with a $\delta^{15}N$ up to −14‰) which is not resolved by pyrolysis (Fig. 4*a*). Another oxidatively treated residue, Murchison 2C10m, physically different from 2C10f in grain size (1 to 3 μm), is clearly distinct on the basis of its nitrogen. The amounts of nitrogen were about an order of magnitude smaller so that isotope ratio measurements were only possible for two combustion steps covering 92% of the total nitrogen, at 500 and 600 °C. Although this is the range in which light nitrogen appears (Fig. 4*b*), neither fraction showed evidence of it, the $\delta^{15}N$ values being +17‰ and +49‰. There is a further well resolved high temperature component of nitrogen at 1,100 °C in 2C10m which might correspond to the heavy nitrogen predicted from bulk Murchison and combustion of 2C10f but no

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Table 2 Correlations of anomalous components

Sample	C (%)	N (p.p.m.)	C_{heavy} (p.p.m.)	N_{light} (p.p.m.)	Xe^{136}_{CCF}	Xe_s^{130}	Ne-E(L)
					($\times 10^{-10}$ cc STP g^{-1})		
Murchison 2C10 f	65.0	5,190	4,500	3,800	420	1.90	≤13
Murchison 2C10 m	3.46	293	1,300	<23	0.73	0.66	16
Ratio f/m	19	18	3.5	>170	570	2.9	≤0.8
Allende B1B	49.4	>904	—	>780	860	—	—
Allende BB	79.3	>906	—	>310	180	—	—
Ratio B1B/BB	—	—	—	2.5	4.7	—	—

reliable isotopic measurements could be obtained on sub-nanogram amounts of gas.

Light nitrogen

Evidently chemical treatments (Table 1) have succeeded in enriching a distinctive, light nitrogen component in the most processed samples. The lowest $\delta^{15}N$ value of −326‰ approaches the −500‰ postulated to exist in Allende[5] and lunar surface materials[22], and called 'light planetary nitrogen' by Geiss and Bochsler[22]. Such extreme compositions cannot be achieved by means of isotopic fractionation under plausible solar system conditions, and apparently require thermonuclear reactions in stars. There is evidence for isotopically light nitrogen in lunar regolith materials which some authors attribute to a secular variation in the solar wind[24,25]. However, since no universally accepted mechanism is able to account for such changes in the Sun without other elements being affected, the subject remains controversial[22]. In principle, ion–molecule reactions in cold interstellar clouds could produce large isotopic fractionations[26] of nitrogen, but these would be of the wrong sign and affect only small molecules. Large molecules made by polymerization of small molecules inherit the same anomalies and so ion-molecule reactions are a distinct possibility for producing unusual compositions in organic polymeric material. We must stress, however, the acid and oxidation residues dealt with here are elemental, not organic carbon, and hence can be made from organic precursors only by charring. Conditions for charring (~600 K and absence of H_2, H_2O and so on) are not met in any astrophysical environment we know of, except small bodies in the solar nebula. Nitrogen, unlike Xe, is a major consituent of Allende carbon (atom fraction 10^{-10} compared with 10^{-2}) and hence must be chemically bound somehow rather than being trapped at rare, highly retentive sites[27].

Isotopically light nitrogen is rather common in the Universe, as ^{14}N forms copiously in the CNO cycle, whereas ^{15}N is produced only in rarer events. For further clues to the origin of light nitrogen let us compare the amounts of this species and other anomalous components in the various samples. We do not know whether even the most extreme $\delta^{15}N$ value of −326‰ in Allende CC actually represents the pure component, in view of the limited resolution of the analytical technique and the presence of heavier nitrogen in this and all other samples. However, for quantitative comparisons, we can calculate nominal amounts of light nitrogen in our samples by assuming their nitrogen is a binary mixture of the two most extreme compositions seen in the acid resistant residues −326‰ and +49‰ (Table 2). The calculated amount of light nitrogen (N_{light}, Table 2) in Murchison 2C10m is only an upper limit, as there is no clear evidence for light nitrogen in this sample. Both N_{light} concentrations for Allende B1B and BB are lower limits, as the data used for the calculation were obtained by the combustion technique.

The best clue to the other anomalies associated with light nitrogen is the concentration ratio for the two Murchison samples (Table 2). The finer fraction is greatly (>170×) enriched in light nitrogen and of the anomalous noble gas components only $^{136}Xe_{CCF}$ shows a similar enrichment (570×). The other two components—s-Xe and Ne-E(L)—are enriched by much smaller factors (3–4×) and are therefore thought[3,16] to be related to heavy C which is similarly enriched (3.5×).

The carbon component associated with light nitrogen and CCF-Xe is distinctive but commonplace, with $\delta^{13}C$ reaching only −32 to −38‰ in Allende B1B[3]. Again the pure component may be somewhat lighter, as small amounts of the other types of carbon were present that may not have been fully resolved by the stepped combustion technique.

Nucleosynthetic origin of CCF xenon

The origin of CCF-Xe has remained unsettled for more than a decade with opinions divided between fission of an extinct super-heavy element[28–31] and nucleosynthesis in a supernova[32,33]. No definitive test is available so far, but the association of CCF-Xe with $\delta^{15}N = -326$‰ just demonstrated argues strongly for a nucleosynthetic origin, if indeed N and CCF-Xe belong to the same phase. A second crucial experiment[34], which was performed simultaneously with our nitrogen isotope investigation and also rules out *in situ* fission, is the demonstration that fission-produced Ba, Nd and Sm are absent from samples enriched in CCF-Xe. Nonetheless, many of the objections to a nucleosynthetic origin remain[35,36]. For example, the conditions required to explain the Xe anomalies (explosive nucleosynthesis at $T = 2.0$ to 2.5×10^9 K) are incompatible with the survival of associated lighter elements (He, Ne, Ar, C) of mainly normal isotopic composition. Perhaps several sources and more than one carrier phase will have to be invoked.

Other types of nitrogen

The most highly processed samples, Allende CC and Murchison 2C10f, both show a trend towards isotopically heavier nitrogen at higher temperatures after the main release of light nitrogen (Figs 2, 4). The $\delta^{15}N$ values are unexceptional, being only +34‰ and −14‰. In view of the large temperature steps and hence low resolution of the technique, there is still a chance that a small heavy nitrogen component exists but is diluted by a residual fraction of more abundant light nitrogen. Heavy nitrogen could be expected to be associated with heavy carbon, which also evolves at high temperatures[3]. Against this argument, it is significant that heavy carbon from Murchison 2C10f (4,500 p.p.m.) appears only in the last ~1% of the release whereas the postulated heavy nitrogen (930 p.p.m., without allowance for the incomplete yield in combustion) constitutes the final 18% of the gas evolved. If we accept this result at face value, the phase containing heavy nitrogen and carbon has an exceedingly high N/C ratio of 0.21. Species of such high N/C ratios and elevated combustion temperatures are unlikely. Consequently we are inclined to attribute most of the nitrogen in the final 18% to residual light N. Assuming that heavy C has an N/C ratio of 0.008, like bulk Murchison, 2C10f and 2C10m (Table 2), then the associated nitrogen could have a $\delta^{15}N$ as high as +5,900‰. Of course, this value is only illustrative as the assumptions of only two components and a fixed N/C ratio are debatable. But it is interesting that bulk Murchison has rising $\delta^{15}N$ to +153‰ at high temperatures (Fig. 3*a*).

A demonstrable link between ^{15}N and ^{13}C would help to narrow the range of astronomical sources. It is known that substantial amounts of ^{15}N and ^{13}C are produced in novae, which are possible sources of Ne-E[37–39]. On the other hand,

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normal red giant stars, while similarly enriched in ^{13}C, are depleted rather than enriched in ^{15}N. An association between heavy nitrogen and Ne-E(L) would strengthen the case for a nova origin for the progenitor (^{22}Na) of the neon gas as already argued by Arnould and Norgaard[39]. Present resolution clearly is not adequate to resolve the nitrogen component associated with s-process Xe. The nitrogen ought to be isotopically light, as expected for a red giant, and so may in part off-set the heavy nitrogen associated with Ne-E.

We thank NASA (NGL-14-001-010) (Chicago) and SERC (Cambridge) for financial support, L. Alaerts for the separation of Murchison, and members of the P.S.U. for helpful discussions.

Received 30 June; accepted 18 August 1983.

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Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells

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Rat cells transformed by the highly oncogenic adenovirus 12 lack at least two cellular proteins which are present in cells transformed by the non-oncogenic adenovirus 5 and in untransformed cells. One protein has been identified as the heavy chain of the rat class I major histocompatibility complex. This finding may explain the difference in oncogenicity between adenoviral species.

HUMAN adenoviruses are double-stranded DNA viruses some of which are capable of inducing neoplasms in rodents[1]. An interesting aspect of adenoviruses is that their oncogenic potential varies: for example adenovirus 12 (Ad12) is strongly oncogenic and induces tumours at high frequency with a short latency period (1–3 months), while adenovirus 5 (Ad5) is non-oncogenic. Virus or isolated DNA of all serotypes, however, can transform rodent cells *in vitro*[1–3]. The tumorigenicity of the resulting transformed cells in nude mice and syngeneic rats reflects the oncogenic potential of the adenovirus species used for transformation, Ad12-transformed cells being more oncogenic than Ad5-transformed cells[4–7]. The transforming activity of the DNA has been assigned to the left-most 11% (early region I, *E1*) of the viral genome[6,8,9]. This region consists of two transcriptional units, *E1a* and *E1b*[10,11], coding for proteins (T antigens) of molecular weights (MWs) 30–40,000, and 19,000 and 55,000 respectively. The transforming regions of the oncogenic and non-oncogenic species are highly homologous with respect to structural organization and nucleotide sequence[12–14], so the substantial difference in oncogenic potential between serotypes must be due to minor, but decisive, differences between the T antigens coded for by region *E1*.

How the divergence in the viral T antigens affects the oncogenic potential of the adenovirus-transformed cells is unknown. Two major concepts have been put forward: one is that the oncogenic cell might be less immunogenic to the host than its non-oncogenic counterpart; and the other that the oncogenic cell might be resistant to the cellular immune defence of the host, while the non-oncogenic cell is susceptible to it (see ref. 15 for discussion). Differences in oncogenicity between transformed cells may, therefore, be determined by the way the transformed cell interacts with the immune system of the host. This interaction might be mediated by the viral T antigens or by cellular proteins which are modulated by viral antigens. The modulation can be induction or suppression of proteins resulting in either rejection or acceptance of the non-oncogenic or oncogenic cells respectively. Such a modulation has recently been demonstrated at the level of mRNA transcription in cells transformed by the DNA tumour virus simian virus 40 (ref. 16).

We have now investigated the role of cellular proteins in oncogenicity of adenovirus-transformed cells, using an immunological approach. Highly oncogenic adenovirus-transformed baby rat kidney (BRK) cells and non-transformed BRK cells were compared immunologically by raising antibodies against them in heterologous BALB/c mice. These sera were compared with respect to their reactivity with panels of oncogenic and non-oncogenic transformed cells. We show here that oncogenicity of the transformed cells is specifically correlated with the absence of 32,000- and 45,000-MW cellular proteins (32K and 45K respectively). The latter protein is identified as the heavy chain of the rat class I major histocompatibility complex (MHC).

0028–0836/83/430771—05$01.00

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Suppression of nonviral protein expression

BALB/c mice were immunized with either nontransformed primary kidney cells of 6-day-old Wag-Rij rats or highly oncogenic cells transformed by a plasmid containing the left-terminal *Eco*RI-C fragment of Ad12 DNA (pAd12RIC), which comprises the entire early region 1[13,17,18]. Sera from several mice were used for immunoprecipitation of ^{35}S-methionine-labelled extracts of primary cultures of BRK cells and pAd12RIC-transformed cells. Sera raised against primary rat kidney cells in seven different mice precipitated only a limited number of proteins (Fig. 1, lanes 2–8). A large amount of a 38,000-MW (38K) protein was precipitated from the lysate of primary cells (lanes 2*a*, 4*a*, 8*a*) and was absent in the transformed cell lysate with at least three individual antisera (*b* lanes). Close inspection of the original X-ray film also revealed a 32K protein in untransformed cells which was absent in the transformed cells. When sera raised in BALB/c mice against Ad12-transformed cells were used for immunoprecipitation, no major differences could be observed between proteins precipitated from untransformed and transformed cells (results not shown).

To investigate further which viral genes are involved in the regulation of the 32K and 38K proteins in the transformed cells, a pooled mouse serum was used for an analysis of a panel of adenovirus-transformed cell lines. The 32K protein is also present in a cell line transformed by a plasmid containing region *E1* of Ad5 DNA (pAd5*Xho*IC; Fig. 2), which suggests that Ad12 sequences suppress the expression of the 32K protein after transformation of the rat cells whereas Ad5 sequences do not. Rat cells transformed by Ad12 region *E1* plasmids in which either the 19K (ref. 19) or the 54K (ref. 6) *E1b* proteins are truncated due to frameshift mutations also lack the 32K protein (Fig. 2, lanes 3, 6). This indicates that the suppression of the 32K protein is not a function of the Ad12 *E1b* proteins but, more likely, an effect of the Ad12 *E1a* region. Thus Ad5 region *E1a* would lack this property.

It could be argued, however, that Ad5 region *E1a*, like Ad12 *E1a*, switches off the expression of the 32K protein, but that the Ad5 *E1b* proteins antagonize this effect. To exclude this possibility, Ad5-transformed cells lacking either the 21K or 55K *E1b* proteins, or both, were investigated. All three transformed cell lines produced the 32K protein (Fig. 2, lanes 7, 4 and 5 respectively), indicating that the Ad5 *E1b* proteins are not involved in the regulation of the expression of 32K. This is further supported by experiments with cell lines transformed by hybrid plasmids[17] consisting of Ad5 *E1a* and Ad12 *E1b* (pAd512), and vice versa (pAd125). These experiments also show (Fig. 2, lanes 8, 9) that the absence of the 32K protein is correlated with the presence of Ad12 region *E1a*, even when the *E1b* sequences originate from Ad5.

As discussed in the accompanying paper[18], cells containing Ad12 region *E1a* are oncogenic in immunocompetent rats, while cells containing Ad5 *E1a* are not. Since only cells expressing Ad12 *E1a* sequences lack the 32K protein, the 32K protein may be involved in the rejection of non-oncogenic adenovirus-transformed cells.

There is no direct correlation between the occurrence of the 38K protein and the presence of Ad5 *E1a* sequences (Fig. 2).

38K protein and β_2-microglobulin

To investigate whether the 32K protein is a membrane protein, cells were labelled with radioactive iodine and extracts of the cells were immunoprecipitated with the pooled BALB/c anti-BRK serum used in the previous experiment. No 32K protein could be precipitated from primary and transformed cells (Fig. 3, lanes 1*b*, 2*b*), which suggests that the protein is probably not a membrane protein. Very prominent 38K and 12K proteins, however, were precipitated from primary cells, but not from Ad12-transformed cells. Since several membrane proteins of 40–45,000 MW and coded for by the class I MHC are known to be associated with a small 12K molecule, β_2-microglobulin[20–22], we used the same ^{125}I-labelled cell extract for

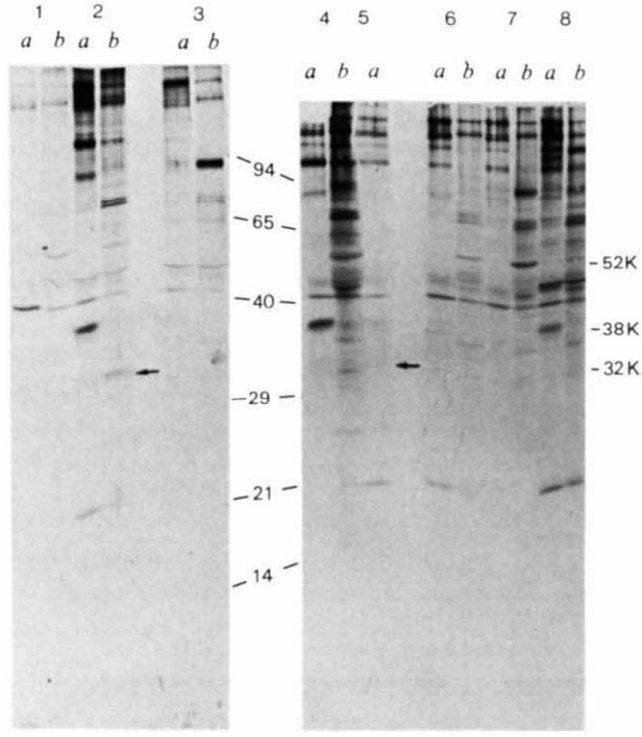

Fig. 1 SDS–polyacrylamide gel electrophoresis of proteins immunoprecipitated from extracts of ^{35}S-methionine-labelled cells with antisera raised against primary BRK cells. Antisera were prepared by immunization of 10-week-old BALB/c mice with 10^7 cells of cultures of primary BRK cells derived from 6-day-old Wag-Rij rats (a highly inbred Wistar strain obtained from the Radiobiological Institute, Rijswijk). Three intraperitoneal injections were given at 10-day intervals; the first injection was combined with complete Freund's adjuvant. The mice were bled 14 days after the last injection. Cell-labelling and subsequent immunoprecipitation procedures have been described previously[17]. Antisera from seven different mice (lanes 2–8) were used for immunoprecipitation of extracts of primary BRK cells (lanes *a*) or extracts of cells transformed by a plasmid containing Ad12 early region *E1* (pAd12RIC)[13] (lanes *b*). The molecular weight of several marker proteins are indicated. Lane 1 represents a control precipitation with normal mouse serum.

immunoprecipitation with a commercial antiserum against human β_2-microglobulin, which cross-reacts with rat β_2-microglobulin chains (our unpublished results). A considerable amount of β_2-microglobulin was precipitated from primary BRK cells (Fig. 3, lane 3*d*) and Ad5-transformed cells (lane 4*d*) but the amount precipitated from Ad12-transformed cells was substantially reduced (lane 5*d*). No heavy-chain class I MHC molecules were co-precipitated with β_2-microglobulin. This might be explained by the fact that the antiserum only reacts with free β_2-microglobulin molecules because it only recognizes the evolutionarily conserved domain which may be shielded when the molecule is complexed to the heavy chain. These free chains may have been dissociated from the MHC heavy chain as an artefact of the immunoprecipitation procedure after extraction of the complex from the membrane. The strong reduction of the amount of β_2-microglobulin on the membrane of Ad12-transformed cells suggests also that the expansion of class I MHC molecules on the surface of these cells might be suppressed.

Class I MHC molecule expression

The presence of class I MHC antigens on the cell surface of adenovirus-transformed rat cells was further investigated using specific rat alloantisera. The rat MHC, the *RT1* complex, comprises five loci coding for histocompatibility antigens. Two loci, *RT1.A* and *RT1.E*, control the expression of the classical serologically detectable class I antigens[23]. All primary and trans-

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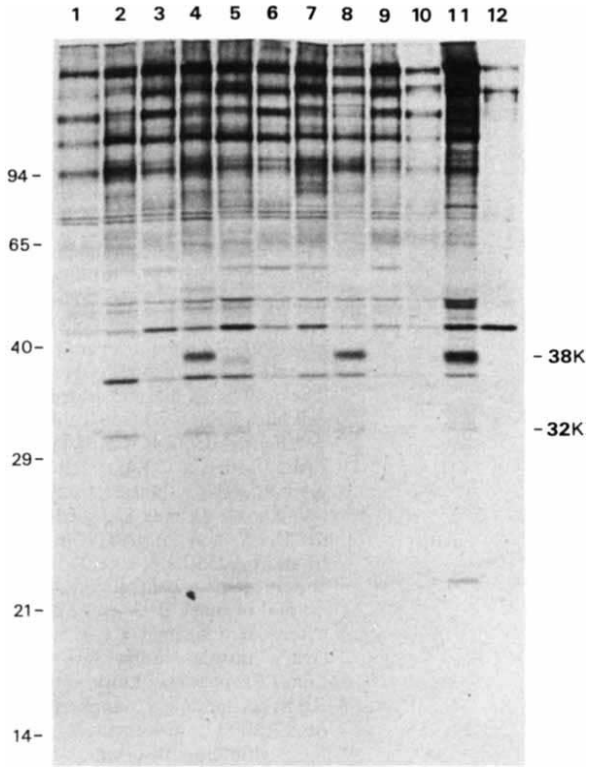

Fig. 2 SDS–polyacrylamide gel electrophoresis of proteins immunoprecipitated from extracts of ^{35}S-methionine-labelled adenovirus-transformed cell lines with mouse antiserum raised against primary BRK cells. The antisera for lanes 2, 4, 5 and 8 (see Fig. 1 legend) were pooled and used for immunoprecipitation of rat cell lines transformed by the following plasmids (see Table 1 for expression of viral genes in the transformed cells): lane 1, pAd12RIC; 2, pAd5*Xho*C; 3, pAd12*Hin*dIIIG; 4, pAd5*Hin*dIIIG; 5, pAd5*Hpa*IE; 6, pAd12dl*Acc*; 7, pAd5dl*Sac*; 8, pAd512; 9, pAd125; 10, primary BRK cells; 11, primary BRK cells precipitated with a normal mouse serum.

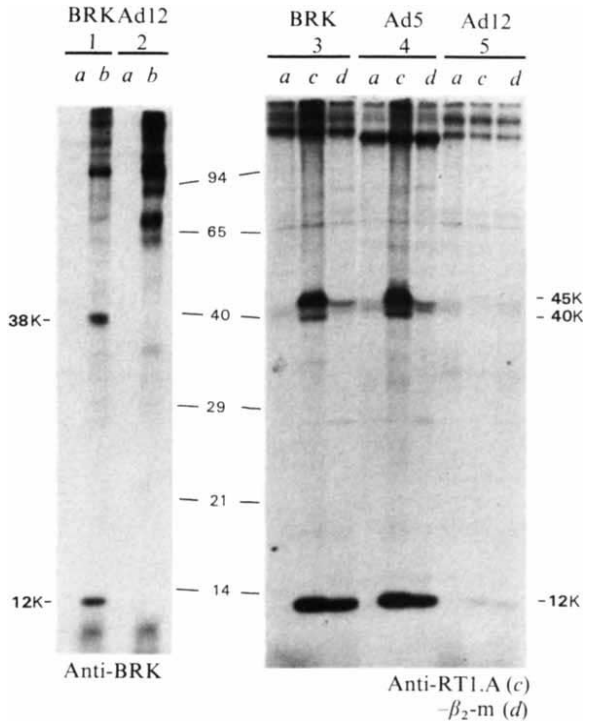

Fig. 3 SDS–polyacrylamide gel electrophoresis of proteins immunoprecipitated from extracts of ^{125}I-labelled cells with mouse anti-primary BRK antiserum, an anti-Wag-Rij alloantiserum and a heteroantiserum against β_2-microglobulin. Monolayer cultures of the cells were washed with phosphate-buffered saline (PBS) and scraped off the plates with a rubber policeman. 10^7 cells in 1 ml of PBS were transferred to an Iodogen-coated Pyrex glass tube and 1 mCi of Na^{125}I was added[33]. After incubation at room temperature for 15 min, the cells were washed with PBS and extracted with immunoprecipitation buffer[17]. Extracts of the following cells were used for immunoprecipitation: lanes 1, 3, primary BRK cells; 2, 5, pAd12RIC-transformed BRK cells; 4, pAd5*Xho*C-transformed BRK cells. The antisera used were a normal mouse antiserum (lanes *a*), the mouse anti-primary BRK antiserum (see Fig. 2 legend), a Lewis anti-Wag-Rij hyperimmune rat alloantiserum (lanes *c*) and an anti-human β_2-microglobulin antiserum (anti-β_2-m; Dako; lanes *d*).

formed rat kidney cells described in this study originate from Wag-Rij rats, a highly inbred Wistar strain, carrying the *RT1*^{u} haplotype. Hyperimmune sera against this haplotype raised in Brown Norway (*RT1*^{n}) or Lewis (*RT1*^{l}) rats precipitated almost exclusively 45K and 12K proteins from ^{125}I-labelled primary BRK cells (Fig. 3, lane 3*c*), representing the *RT1.A* heavy chain and β_2-microglobulin respectively (compare with lane 3*d*). A similar amount of *RT1.A* was found in cells transformed by Ad5 *E1* DNA (Fig. 3, lane 4*c*). However, cells transformed by Ad12 *E1* DNA contained a greatly reduced amount of the 45K protein (Fig. 3, lane 5*c*). Similar results were obtained when cells were metabolically labelled with ^{35}S-methionine and subsequently precipitated with the Lewis anti-Wag-Rij serum (Fig. 4*A*, lanes 1*b*, 3*b*, 8*b*).

Suppression of *RT1.A* by Ad12 *E1a*

To investigate which viral gene determines the suppression of *RT1.A*, a panel of transformed cells was analysed by immunoprecipitation with the anti-*RT1.A*^{u} antiserum. Only cells containing the Ad12 *E1a* region had strongly reduced amounts of *RT1.A* (Fig. 4). All cells containing the *E1a* region of Ad5, including those harbouring Ad12 *E1b* sequences, expressed normal levels of *RT1.A* (Fig. 4A, lanes 1–3, 7). Interestingly, suppression of *RT1.A* parallels that of the 32K protein; thus oncogenicity of adenovirus-transformed cells in syngneic immunocompetent rats is strictly correlated with the suppression of the class I MHC antigens and of the 32K protein. These effects are specifically induced by Ad12 *E1a*, but not Ad5 *E1a*, region.

The almost complete absence of alloreactive MHC class I molecules on the surface of Ad12-transformed cells may have several causes. To test whether the phenomenon is due to the lack of β_2-microglobulin as is the case in Daudi cells[24], we investigated metabolically labelled cells for the presence of β_2-microglobulin. From ^{35}S-methionine-labelled Ad12-transformed cells, which lack the *RT1.A* heavy chain completely, a normal amount of β_2-microglobulin can be precipitated (Fig. 4*B*, lane 2*b*), as compared with Ad5-transformed cells (Fig. 4*B*, lane 2*a*). Thus, the absence of *RT1.A* on the membrane of Ad12-transformed cells is not due to a lack of β_2-microglobulin synthesis. A second possibility was that the absence of MHC class I molecules on the cell surface can be explained by the appearance of 'alien', 'extra' or 'inappropriate' MHC specificities on the cell surface as has been reported elsewhere[25,26]. The mechanism of these phenotypic switches is not known. Furthermore, the loss, or alteration, of allogeneic reactivity on tumour cells may be the result of structural alterations of the class I molecule due to association with products of viral replication or expression[27,28].

To exclude these possibilities the expression of MHC class I-specific mRNA from several adenovirus-transformed cell lines was examined using a cloned DNA probe[29] containing sequences of the human *HLA B7* gene (Fig. 5). Since the probe contains sequences of the constant (third) domain, it should react with

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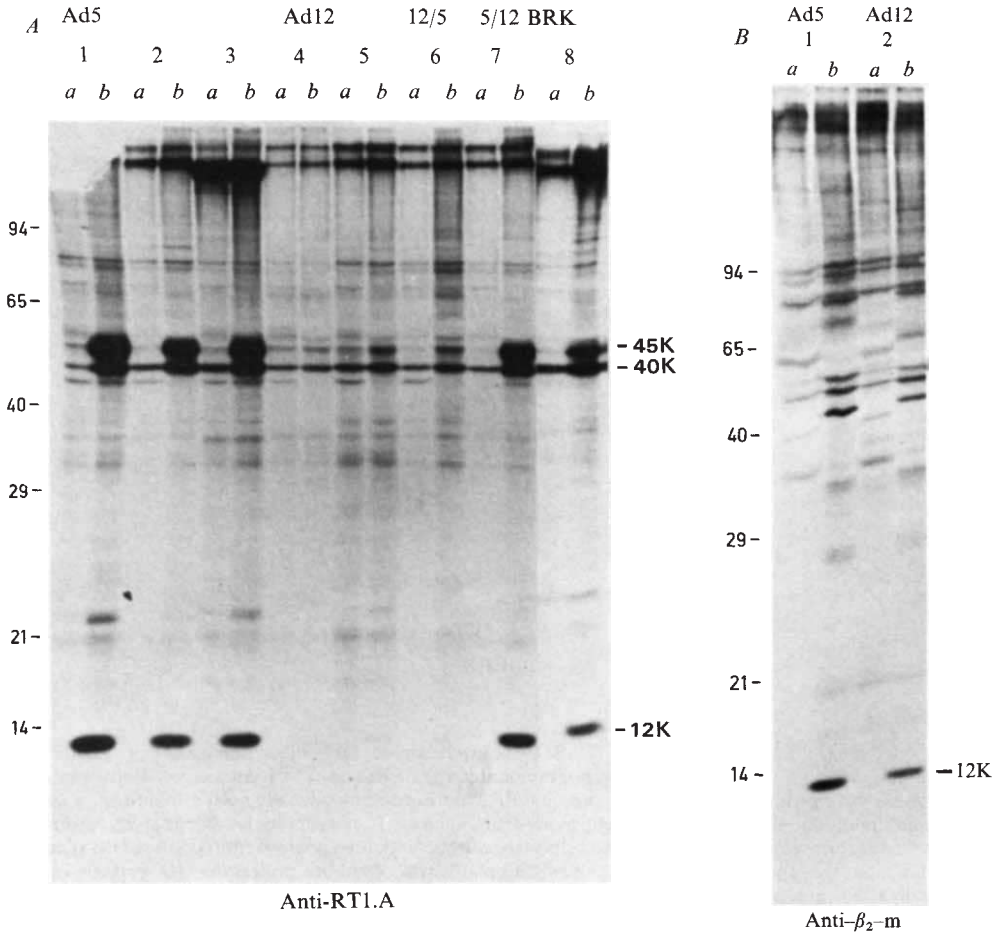

Fig. 4 SDS–polyacrylamide gel electrophoresis of proteins immunoprecipitated from extracts of ^{35}S-methionine-labelled cells with an anti-Wag-Rij alloantiserum (*A*) and an anti-β_2-microglobulin serum (*B*). The following plasmid-transformed cell lines were used (see Table 1 for details): pAd5*XhoC* (*A*, *B*, lanes 1); pAd5*Hin*dIIIG (*A*, lane 2); pAd5*Hpa*IE (*A*, lane 3); pAd12RIC (*A*, lane 4; *B*, lane 2); pAd12*Hin*dIIIG (*A*, lane 5); pAd125 (*A*, lane 6) and pAd512 (*A*, lane 7). *A*, lane 8, represents a control consisting of normal primary BRK cells. Lanes *a* represent a normal rat serum (*A*) and a normal rabbit serum (*B*). Lanes *b* represent a Lewis anti-Wag-Rij hyperimmune alloantiserum (*A*) or a rabbit anti-human β_2-microglobulin antiserum (*B*).

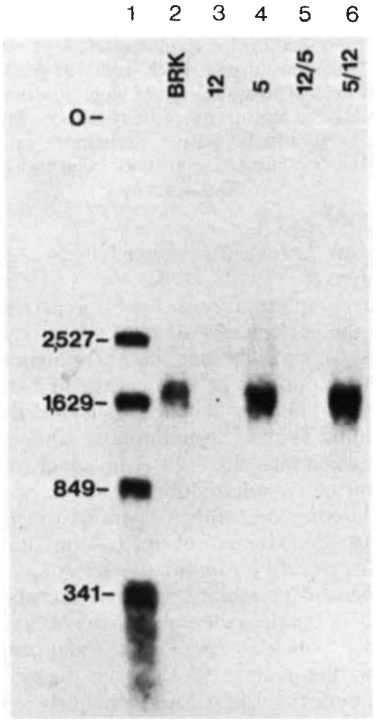

Fig. 5 Class I MHC transcripts in primary and adenovirus-transformed BRK cells. Cytoplasmic RNA was isolated by resuspending a cell pellet in 10 mM Tris-HCl (*p*H 7.8), 1 mM EDTA, 150 mM NaCl, 0.65% NP40 at 4 °C. The nuclear pellet was removed, the supernatant extracted with phenol–chloroform and the RNA precipitated in ethanol. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography[34]. One μg of poly(A)$^+$ RNA (selected once) was separated by electrophoresis in 1.0% agarose–formaldehyde gels[35] for 4 h at 7.5 V cm^{-1} and thereafter blotted overnight onto a nitrocellulose filter in a buffer consisting of 16×SSC, 2.2 M formaldehyde. The blot was baked for 2 h at 80 °C and prehybridized for at least 3 h at 42 °C in a buffer containing 0.75 M NaCl, 50 mM sodium phosphate (*p*H 7.4), 5 mM EDTA, 0.1% SDS, 5×Denhardt solution (0.1% each of Ficoll, bovine serum albumin and polyvinylpyrrolidone), 0.25 mg denatured calf thymus DNA per ml and 50% formamide. Subsequently, the blot was hybridized for 16 h at 42 °C in the same buffer, containing 5×10^6 c.p.m. ml^{-1} of a nick-translated human *HLA B7* cDNA probe[29], washed three times in 2×SSC, 0.1% SDS at 50 °C for about 2 h and autoradiographed for 40 h. Rat cell lines transformed by the following plasmids were used: lane 2, primary BRK cells; 3, pAd12RIC; 4, pAd5*XhoC*; 5, pAd125; 6, pAd512. Lane 1 represents a 5′-end-labelled *Hae*III digest of phage M13 mp8 DNA (molecular weights are given in base pairs). O, origin.

any MHC class I heavy-chain mRNA present in rat cells. Figure 5, lane 2, shows that the probe hybridizes to a 1.7-kilobase (kb) mRNA present in RNA isolated from normal BRK cells. This band probably represents the RNA coding for the *RT1.A* heavy chain. The amount of MHC class I-specific mRNA is greatly reduced in transformed cells harbouring Ad12 *E1a* sequences, irrespective of the identity of the *E1b* region (Fig. 5, lanes 3, 5). In contrast, transformed cells containing the Ad5 *E1a* region do express normal amounts of *RT1.A* mRNA, again independent of the identity of the *E1b* region (lanes 4, 6). These results clearly demonstrate that the absence of *RT1.A* antigenic determinants in Ad12-transformed cells is due to a mechanism regulating the mRNA production, rather than modifying the antigenic properties of the *RT1.A* molecules.

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Table 1 Proteins present in a number of adenovirus-transformed rat cell lines with different oncogenic potential

Adenovirus-transformed rat cell line	Expression of Ad5 or Ad12 region *E1a*	*E1b*	Protein present in cellular extract 32K	*RT1.A*^{u} (45K)	β_2-microglobulin (12K)
BRK (untransformed)	—	—	+	+	+
pAd5*Xho*IC[17]	5	5	+	+	+
pAd5*Hin*dIIIG[19]	5	5(19K)	+	+	+
pAd5dl*Sac*[19]	5	5(58K)	+	+	+
pAd5*Hpa*IE[17]	5	—	+	+	+
pAd12RIC[13]	12	12	−	−	+
pAd12*Hin*dIIIG[6]	12	12(19K)	−	−	+
pAd12dl*Acc*[19]	12	12(54K)	−	−	+
pAd512[17]	5	12	+	+	+
pAd125[17]	12	5	−	−	+

+, Present; −, absent or strongly reduced.

Discussion

In an attempt to understand why Ad12-transformed cells are capable of escaping the immune defence of immunocompetent rats, we investigated possible differences in the proteins contained in various oncogenic and non-oncogenic adenovirus-transformed cells (Table 1). Two proteins, normally present in untransformed and Ad5-transformed cells, were absent from Ad12-transformed cells. One of these proteins has a molecular weight of 32,000, while the other was identified as the heavy chain (45K) of the rat class I MHC *RT1.A*. The light chain, β_2-microglobulin, was present in normal amounts in Ad12-transformed cells and the suppression of the 45K protein was shown to occur at the level of mRNA synthesis. The disappearance of both the 32K and 45K proteins is strictly correlated with the presence of Ad12 region *E1a*, and is totally independent of the identity of the *E1b* region (Table 1). From tumorigenicity data, it can be concluded that oncogenicity of the transformed cells in immunocompetent rats is also strictly correlated with the expression of Ad12 *E1a* sequences[18]. Thus, the essential question is whether the reduced level of class I MHC molecules can account for the high oncogenicity of Ad12-transformed cells. Our accompanying article[18] shows that rejection of transformed cells is probably an activity of cytotoxic T cells (CTLs) from which Ad12 *E1a*-transformed cells are able to escape. CTL activity, like a number of cell–cell interactions in the immune system, is mediated via antigens encoded in the MHC, whose presence on the cell surface is therefore necessary[30,31]. Consequently, tumour cells expressing viral antigens, but not class I MHC antigens, cannot be killed through T-cell cytotoxicity[31]. This phenomenon, generally known as 'MHC restriction', can explain the resistance of the class I MHC-deficient Ad12-transformed cells against the cellular immune response of the host, resulting in growth into a tumour. Experiments showing the deficiency of a CTL response to Ad12-transformed cells *in vitro* are published in our accompanying paper[18].

The possibility cannot be excluded that Ad12 only tranforms target cells which lack expression of class I MHC genes. Experiments to test this possibility are in progress.

To our knowledge, the findings reported here represent the first demonstration of an interaction of a viral gene product (that of Ad12 *E1a*) with the control of a specific cellular gene which appears to influence the oncogenic potential of the cells. Our conclusions are confirmed by a recent report[32] demonstrating that SV40-transformed mouse C3H fibroblasts, which were selected for high oncogenic potential in syngeneic mice by serial passage, had lost expression of the *H-2K*^{k} allele of the murine class I MHC. These highly oncogenic cells were resistant to killing by secondary anti-SV40 CTLs, in contrast to non-oncogenic cells which were lysed, unless the reaction was blocked with anti-*H-2K*^{k} antibodies. Taking all the data together, we conclude that the MHC class I-restricted CTL response may represent an important mechanism for *in vivo* rejection of DNA tumour virus-transformed cells, and that oncogenicity may result from suppression of class I major histocompatibility antigens.

We thank Drs L. C. Paul and L. Vaessen for generous gifts of rat alloantisera and Dr S. M. Weissman for the cloned cDNA probe coding for HLA B7. The work was supported in part by the Dutch Foundation for Pure Research (ZWO) through the Foundation for Fundamental Medical Research (FUNGO).

Received 16 June; accepted 2 September 1983.

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Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity

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Evidence is presented that cells transformed by adenovirus type 12 are oncogenic because they escape from T-cell immunity. This effect is brought about by reducing the expression of class I transplantation antigens and is a function of the protein translated from the 13S mRNA, transcribed from early region 1a. *These findings establish a novel mechanism by which transformed cells can acquire an oncogenic phenotype.*

ADENOVIRUS-transformed rodent cells exhibit variable degrees of oncogenicity, depending on the adenovirus species used for transformation: rat cells transformed by the highly oncogenic adenovirus 12 (Ad12) are highly oncogenic in the syngeneic host[1], whereas rat cells transformed by the non-oncogenic adenoviruses, for example Ad2 and Ad5, are rarely tumorigenic in immunocompetent syngeneic rats[2]. It has been proposed that cells transformed by the non-oncogenic adenoviruses fail to form tumours because they are more antigenic than are cells transformed by oncogenic adenoviruses and therefore are more readily eliminated by the immune system of the host[3]. The main evidence supporting this hypothesis is that rat cells transformed by the non-oncogenic Ad2, which are non-oncogenic in immunocompetent rats, do form tumours in immunosuppressed rats[4] and in congenitally athymic nude mice[5].

The adenovirus genes responsible for transformation *in vitro* are localized in a region near the left end of the viral genome, in a 4-kilobase (kb) DNA segment which comprises the first region of the genome to be expressed in lytic infection, and therefore designated early region *1* (*E1*). This region consists of two transcriptional units, *E1a* and *E1b* (ref. 6). We have recently demonstrated that the difference in oncogenic potential between Ad5- and Ad12-transformed cells in nude mice is determined by the large tumour antigen specified by subregion *E1b*[7,8]. We show here that the identity of the *E1a* subregion determines the susceptibility of transformed cells to the cellular immune system, that is, rat cells expressing Ad5 *E1a* are highly susceptible to cytotoxic T cells and so are only oncogenic in immunodeficient animals, whereas cells expressing Ad12 *E1a* have a reduced susceptibility for T-killer cells and hence are oncogenic. By using a set of cell lines transformed by mutant *E1* regions we show that the inactivation of major histocompatibility complex (MHC) class I gene expression is a function of the product of the Ad12 13S *E1a* mRNA.

Oncogenicity of Ad-transformed cells

We have recently reported the construction of two Ad5–Ad12 hybrid early region *1* plasmids: one, pAd512, consisting of the *E1a* region of non-oncogenic Ad5 and the *E1b* region of highly oncogenic Ad12; and the other, pAd125, consisting of Ad12 *E1a* and Ad5 *E1b*. These plasmids were used to transform primary baby rat kidney (BRK) cells and the oncogenicity of the resulting transformed cell lines was tested in nude mice[7]. Comparison of the oncogenic potential of the various transformed cell lines in immunocompetent rats and in immunodeficient nude mice (Table 1) revealed a major contribution of region *E1a* in oncogenicity: cells expressing Ad5 *E1a* (Ad5 *E1*- and pAd512-transformed cells) were both oncogenic in nude mice, but completely failed to induce tumours in immunocompetent rats. On the other hand, cells expressing Ad12 *E1a* (Ad12 *E1*- and pAd125-transformed cells) manifested a similar oncogenic potential both in immunodeficient nude mice and in immunocompetent rats. The different results obtained in the mouse and rat assay systems for oncogenicity are most readily explained in terms of a thymus-mediated immune response, which is present in the immunocompetent rat but absent in the nude mouse. The finding that pAd512-transformed cells were also highly oncogenic in immunodeficient nude rats (Table 1) is consistent with this explanation. Therefore, these data suggest that cells expressing Ad5 *E1a* are susceptible to elimination by T cells and so are non-oncogenic in the immunocompetent rat, in spite of their limited (Ad5 *E1*) or high (Ad512) oncogenicity in nude mice. On the other hand, cells expressing Ad12 *E1a* seem to escape

Table 1 Oncogenicity of adenovirus-transformed cells in syngeneic rats

Plasmids used for transformation	Expression in transformed cell		% Oncogenicity of transformed cell in		
	E1a	*E1b*	Nude mice	Syngeneic rats	Nude rats
pAd5*Xho*C	5	5	50 (15/31)	0 (0/51)	ND
pAd12RIC	12	12	100 (23/23)	100 (18/18)*	ND
pAd512	5	12	100 (18/18)	0 (0/26)	100 (6/6)‡
pAd125	12	5	10 (2/19)	10 (6/60)†	ND
p51212	5+12	12	100 (12/12)	0 (0/18)	ND

10^7 transformed cells were injected subcutaneously into 4-day-old Wag-Rij rats or in adult athymic nude Wag-Rij rats. The cell lines used and the oncogenicity of these lines in athymic nude mice have been described before[7], with the exception of p51212-transformed cells, which express the Ad5 and Ad12 *E1a* regions simultaneously at comparable levels plus the Ad12 *E1b* region (Fig. 3). For each type of transformed cells at least three independently isolated cells lines were used. Numbers in parentheses indicate the total number of animals with tumours/total number of animals injected. Average latent periods were: *, 6 weeks; †, 4 months; ‡, 3 months. ND, not done.

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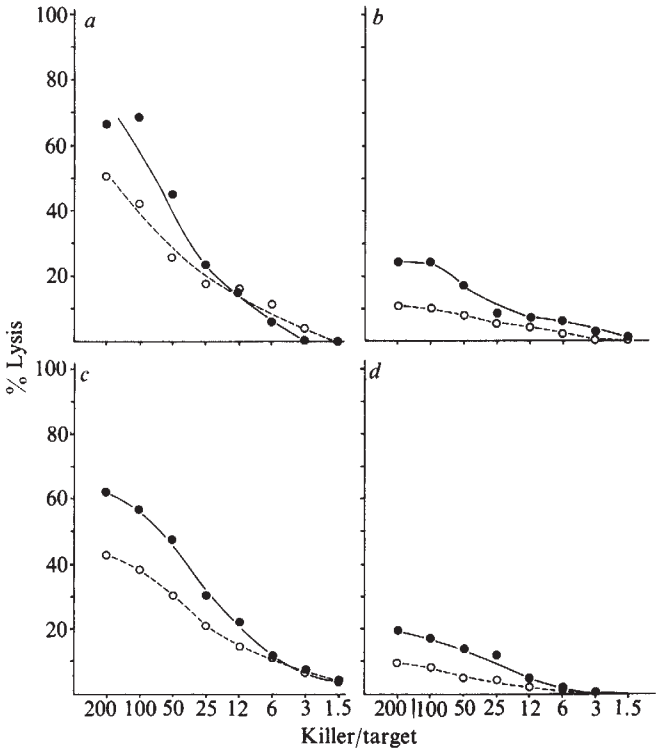

Fig. 1 Susceptibility of adenovirus-transformed Wag-Rij cells to allogeneic CTLs. For every type of transformed target cell, the lytic activity of CTLs is shown against two independently derived cell lines. *a*, Cells transformed by Ad5 region *E1*, pAd5*XhoC* (ref. 7). *b*, Cells transformed by Ad12 region *E1*, pAd12RIC (ref. 26). *c*, Cells transformed by a plasmid containing Ad5 *E1a* and Ad12 *E1b*, pAd512 (ref. 7). *d*, Cells transformed by a plasmid containing Ad12 *E1a* and Ad5 *E1b*, pAd125 (ref. 7). Target cells were labelled with 100 μCi of ^{51}Cr-labelled sodium chromate for 30 min at 37 °C, and washed three times to remove free chromium. To raise anti-*RT1^u* allogeneic CTLs, spleen cells of ACI rats (*RT1^a* haplotype) were cultured for 6 days with irradiated (2,500 rad) lymphocytes derived from the spleens of Wag-Rij rats at a ratio of three responders to one stimulator as described previously[27]. Reactivity of CTL was assayed by measuring ^{51}Cr release of triplicate samples of targets in microtitre plates for 4 h at 37 °C, using 10^4 targets per well. Per cent lysis was calculated according to $(E-C)/(M-C)\times 100$, where E = counts released from targets in the presence of CTLs, C = counts released from targets in the absence of CTLs, M = counts released from targets lysed with 5% Triton.

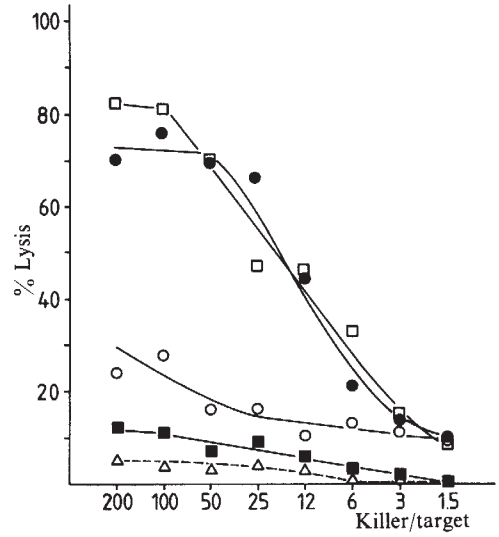

Fig. 2 Reactivity of anti-*RT1^n* allogeneic CTL against Ad5- and Ad12-transformed Brown Norway rat cells. □, ●, Ad5-transformed cell lines; ○, ■, Ad12-transformed cell lines; △, Ad12-transformed Wag-Rij line. Anti-*RT1^n* killer cells were generated in 6-day mixed lymphocyte cultures. Responder strain was the Wag-Rij rat. Stimulators were irradiated Brown Norway Spleen cells. For further explanatory notes, see Fig. 1 legend.

T-cell-mediated destruction since they are equally oncogenic in the nude mice and in the syngeneic rats.

Susceptibility to cytotoxic T cells

In the accompanying paper[9] we show that rat cells expressing the Ad12 *E1a* region have a reduced expression of the heavy chain of the class I transplantation antigens. As foreign (viral) antigens are only recognized by cytoxic T lymphocytes (CTLs) in the context of MHC class I molecules[10], reduced expression of class I polypeptides could explain the escape of cells expressing Ad12 *E1a* from the T-cell-mediated immune response. To investigate the extent to which reduced expression of class I molecules in cells expressing Ad12 *E1a* reduces susceptibility to CTLs, we have measured lysis of Ad5- and Ad12-transformed cells and of the Ad5–Ad12 recombinant cell lines by allogeneic CTLs generated in primary mixed lymphocyte culture. Such CTLs have been shown to be mainly directed against class I molecules[11–13]. All adenovirus-transformed cells used in the oncogenicity studies were derived from kidneys of the highly inbred strain Wag-Rij (*RT1^u* haplotype). To raise anti-*RT1^u* allogeneic CTLs, spleen cells of ACI rats (*RT1^a* haplotype) were cultured *in vitro* with irradiated spleen cells from Wag-Rij rats. The reactivity of these allogeneic CTLs against a set of adenovirus-transformed cells shows a strong response by the allogeneic CTLs against both Ad5 *E1*-transformed cells (Fig. 1*a*) and pAd512-transformed cells (Fig. 1*c*). The same CTLs have a low, but reproducible, reactivity against both Ad12 *E1*- (Fig. 1*b*) and pAd125- (Fig. 1*d*) transformed cells. These data are in excellent agreement with our studies on oncogenicity of these cells in immunodeficient and immunocompetent animals, from which we conclude that cells expressing Ad12 *E1a* escape T-cell-mediated immune response (Table 1).

A similar difference in susceptibility to allogeneic CTLs was observed for Ad5- and Ad12-transformed Brown Norway rat cells (*RT1^n* haplotype) as for Ad5- and Ad12-transformed Wag-Rij cell lines (Fig. 2). It seems likely, therefore, that suppression of class I antigen expression by Ad12 region *E1a* is, at least in the rat system, a rather general phenomenon. A low, but reproducible, CTL response was found against the Ad12-transformed rat cells derived from the two different inbred rat strains tested. Indeed, long exposure of autoradiographs of immunoprecipitations with anti-class I antisera revealed small amounts of class I antigens in these cells. The low residual activity of the mixed lymphocyte culture-activated cells is therefore probably due to specific CTL activity. The specificity of CTLs was shown by the fact that responder-type target cells were not lysed (Fig. 2) and only low specific lysis of unrelated target cells was detected (not shown).

Suppression of class I antigen production

The Ad12 *E1a* region encodes two co-terminal mRNAs of 12S and 13S respectively, differing only in the amount of RNA removed internally by splicing[14]. To investigate which of these two mRNAs, if not both, encodes the inactivating function, we have investigated the expression of class I transplantation antigens in two cell lines transformed by Ad12 region *E1* plasmids carrying specific mutations in the *E1a* region. The results (Fig. 3) show that expression of class I antigens is not reduced in cells transformed by pSVR7, a mutant plasmid with a single nucleotide deletion affecting the 13S mRNA only[15] (lane 1), or in cells transformed by pSVR11, a mutant plasmid with a 106-base pair (bp) deletion affecting both *E1a* mRNA species (lane 2). Thus the Ad12 gene product responsible for the reduced expression of class I antigens is encoded by the 13S *E1a* mRNA.

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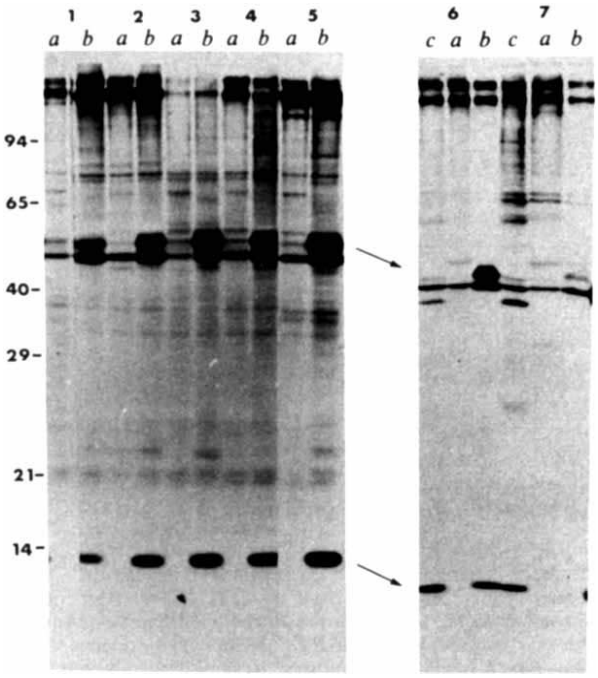

Fig. 3 Immunoprecipitation of ^{35}S-methionine-labelled cell extracts of adenovirus-transformed cells with anti-MHC class I antisera. The sera used were either normal rat serum (lanes *a*) or Lewis anti-Wag-Rij alloantiserum (lanes *b*), or an anti-human β_2-microglobulin antiserum (lanes *c*). The cell lines used were transformed by: pSVR7 (ref. 15), lane 1; pSVR11 (ref. 15), lane 2; pLT12 (ref. 8), lane 3; pST12 (ref. 8), lane 4; p51212, lane 5; p512pm975, lane 6; p512HL1007, lane 7. Positions of the molecular weight markers are indicated. Procedures for immunoprecipitation were as previously described[7]. p51212 was constructed by inserting the Ad12 *Eco*RI C fragment in pAd5 *E1a* (ref. 7). p512pm975 was made by cloning the Ad12 *Eco*RI C fragment into pEKpm975 (ref. 28). p512HL1007 was constructed by inserting a 10-bp *Hin*dIII linker at the single *Sma*I site of pAd5 *E1a* (position 1,007 of the Ad5 sequence) and subsequent insertion of the Ad12 *Eco*RI C fragment in the mutagenized pAd5 *E1a* plasmid. The identity of the mutation was confirmed by DNA sequencing. p512pm975-transformed cells express the Ad12 region *E1* genes plus the Ad5 13S *E1a* mRNA, p512HL1007-transformed cells express Ad12 region *E1* genes, the Ad5 12S *E1a* mRNA gene product plus a truncated 13S *E1a* protein. Correct expression of the mutagenized *E1a* regions in transformed cells was confirmed in S_1 nuclease experiments (not shown).

To investigate the mechanism by which Ad12 region *E1a* switches off the MHC class I genes in transformed cells, we have studied the activity of the genes in cell lines which simultaneously express the *E1a* regions of both Ad5 and Ad12 (ref. 8). The results show that three independently isolated cell lines, co-expressing Ad5 and Ad12 region *E1a*, have normal levels of class I antigens (Fig. 3, lanes 3–5), indicating that expression of Ad5 *E1a* can block the inactivating functions of Ad12 *E1a*. Co-expression of the Ad5 13S *E1a* mRNA and region *E1a* of Ad12 allowed production of class I antigens (see Fig. 3 legend), while cells co-expressing the Ad5 12S *E1a* mRNA and *E1a* of Ad12 did not have detectable levels of class I antigens (Fig 3, lanes 6, 7). Thus, the Ad5 13S *E1a* mRNA is responsible for the dominant effect of Ad5 over Ad12.

Taking advantage of this finding we tested directly the relationship between the reduced class I expression and the highly oncogenic phenotype of the Ad12-transformed cells by using the recombinant plasmid p51212 (see Fig. 3 legend). Rat cells transformed by p51212 express all Ad12 transforming genes plus region *E1a* of Ad5 (not shown). They produce normal amounts of class I antigens (Fig. 3, lane 5), are sensitive to allogeneic CTLs (not shown) and are highly oncogenic in nude mice, but fail to induce tumours in syngeneic rats (Table 1). Thus, cells expressing all Ad12 transforming genes are non-tumorigenic in syngeneic rats if the function which inactivates MHC class I expression is blocked. This strongly suggests that the unique capacity of Ad12 *E1a* virtually to abolish expression of MHC class I genes must be the molecular basis for the difference in oncogenicity between Ad5- and Ad12-transformed cells.

Discussion

Immunological defence mechanisms, mediated by T cells, are considered to play a crucial part in the elimination of virus-induced tumours[13,16,17]. For example, T-cell-deficient nude mice show a markedly increased susceptibility to various virus-induced tumours compared with normal mice[5,18,19]. It has been shown that CTLs only recognize a viral antigen in the context of self-MHC class I antigens[10]. Thus, CTLs have combined specificity for viral antigens and class I MHC molecules, while T-helper cells have combined specificity for antigens and class II MHC molecules. The adenovirus-mediated modulation of MHC class I expression, as described in the accompanying paper[9], offers an ideal system to study the functional role of CTLs in tumour rejection. As a first step we have shown that the decreased expression of class I antigens in Ad12-transformed cells results in a dramatic decrease in susceptibility to allogeneic CTLs, which are known to be directed against class I molecules. Of course, this finding is no direct proof for decreased susceptibility to virus-specific CTLs *in vivo*. However, it seems likely that at least the same amount of class I molecules is required for lysis by syngeneic class I-restricted CTLs as for lysis by allogeneic CTLs, for allogeneic CTLs directly recognize class I molecules, while virus-specific CTLs probably recognize conformational determinants on class I molecules, created by interaction with viral antigens at the cell surface. Indeed, we have found that Ad5-transformed BRK cells induced an excellent adenovirus-specific secondary CTL response *in vitro*, while the same cells transformed by Ad12 practically failed to do so (R.B., unpublished).

Our results indicate that CTLs also have a lytic activity against Ad12-transformed cells. The oncogenic phenotype of Ad12-transformed cells might therefore not be due to a complete absence of CTL reactivity against such cells, but rather to a sufficiently reduced susceptibility to CTLs to allow the cells to evade elimination. Thus, on the basis of the combined *in vivo* and *in vitro* findings we propose that adenovirus-transformed cells expressing the Ad5 *E1a* region are eliminated in the immunocompetent host by an efficient CTL response. Hence, these cells are only oncogenic in immunodeficient animals such as nude mice[5,7], nude rats (Table 1) and immunosuppressed rats[4]. On the other hand, cells expressing Ad12 *E1a* are equally oncogenic in immunocompetent and in T-cell deficient animals (Table 1), apparently because these cells lack sufficient MHC class I antigens in the plasma membrane to be eliminated by specific CTLs (Figs 1, 2).

At this stage we cannot exclude that class II-restricted T cells also have a reduced response against Ad12-transformed cells compared with Ad5-transformed cells. However, both Ad5- and Ad12-transformed cells induce high levels of IgG antibodies in immunized animals. Since production of antibodies requires class II-restricted T-helper cells, Ad12 probably does not interfere with class II-restricted immune response.

We have presented evidence that the gene product of the 13S *E1a* mRNA of Ad12 is responsible for the absence of the heavy chain of class I antigens. We have recently shown that the same Ad12 *E1a* gene product is required to activate the Ad12 *E1b* promoter[20]. A similar activating function has also been demonstrated for the Ad5 *E1a* region, both for activation of region *E1b* and other early regions of the viral genome[21–24], as well as for a cell encoded heat-shock protein[25]. It has been proposed that the *E1a* gene product manifests its activating function through the inactivation of a cellular repressor-like protein[23].

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Therefore, the simplest possible model explaining both the activating and the inactivating functions of the polypeptide encoded by the 13S Ad12 *E1a* mRNA is that it activates a cellular gene (via inactivation of a repressor) which itself has a repressing effect on other cellular genes. However, the Ad12 SVR7 mutant plasmid, which carries a single nucleotide deletion in the region encoding the Ad12 13S *E1a* mRNA, does not switch off the activity of class I genes in transformed cells, but has retained the capacity to activate the Ad12 *E1b* promoter[15]. That we can separate the activating and inactivating functions of the Ad12 *E1a* gene product in the SVR7 mutant suggests that these two functions are not manifested through an interaction with the same cellular control factor.

Finally we have shown that rat cells expressing the Ad5 and Ad12 *E1a* regions simultaneously do express normal levels of class I antigens. Thus, Ad5 *E1a* can prevent Ad12 *E1a* from inactivating the class I genes. Such a dominant effect suggests that Ad5 and Ad12 *E1a* products are competing for binding to the same regulatory factor. The finding that the Ad5 protein responsible for the dominant effect is structurally closely related to the Ad12 product is consistent with such a competitive binding model. Clearly, the next step should be to unravel the mechanism by which the Ad12 *E1a* product interacts with the class I genes. Studies in this direction with cloned murine class I genes are in progress.

This work was supported in part by the Netherlands Organization for the Advancement of Pure Research (ZWO) through the Foundation for Fundamental Medical Research (FUNGO). We thank Dr A. J. Berk for the gift of pEKpm975 and Drs L. C. Paul and L. Vaessen for rat alloantisera.

Received 16 June; accepted 2 September 1983.

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Expression of recessive alleles by chromosomal mechanisms in retinoblastoma

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Inheritance of a mutation at the Rb-1 *locus, which has been mapped to band q14 of human chromosome 13, results in predisposition to retinoblastoma. Cloned DNA segments homologous to arbitrary loci of human chromosome 13 and which reveal polymorphic restriction endonuclease recognition sequences, have been used to look for somatic genetic events that might occur during tumorigenesis. A comparison of constitutional and tumour genotypes from several cases indicates that tumorigenesis may result from the development of homozygosity for the mutant allele at the* Rb-1 *locus. The homozygosity in these cases results from mitotic nondisjunction, resulting in loss of the homologous wild-type chromosome, or from a mitotic recombination event.*

RETINOBLASTOMA is one of several heritable childhood cancers. The disease exhibits both hereditary and sporadic occurrence, with the genetic form inherited as a highly penetrant autosomal dominant trait. Additionally, deletions of human chromosome 13 band q14 are occasionally associated with retinoblastoma[1–3]. Deletion[4] and family studies[5] have shown the genetic locus for the enzyme esterase D (*ES-D*), to be tightly linked to *Rb-1*, the retinoblastoma locus. Additional evidence supporting this map location comes from a family[6] showing a balanced chromosomal translocation in unaffected carrier parents and some unaffected sibs and an unbalanced chromosome 13 deletion in affected individuals. The region of chromosome 13 which was deleted in the predisposed children was q13.1–q14.5, thus including the proposed *Rb-1* locus. This observation indicates that the deletion mutation of the *Rb-1* locus was recessive to two copies of the normal gene. It is possible, then, that the inherited retinoblastoma mutation is actually a recessive allele requiring subsequent inactivation of

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the normal allele on the homologue for its expression in tumorigenesis[7].

It has been proposed that the tumour arises by two mutational steps, because of the observation that while bilateral retinoblastoma is characteristic of the inherited disease, unilateral retinoblastoma is characteristic of the sporadic disease[8–10]. Thus, the hereditary retinoblastomas would arise from a single additional somatic event in a cell which carries an inherited mutation, while sporadic cases would require two somatic events.

Figure 1 outlines specific chromosomal mechanisms which would allow phenotypic expression of a recessive mutant allele at the *Rb-1* locus. The hereditary form of the disease arises as a germinal mutation of the *Rb-1* locus (Fig. 1*a*) and is inherited by an individual who thus carries such a mutation in all of his somatic and germ-line cells (Fig. 1*b*). Any subsequent event in such a predisposed retinal cell which results in homozygosity for the mutant allele (that is, mutant at the *Rb-1* locus on both chromosome 13 homologues) will result in a tumour clone. Possible chromosomal mechanisms during this process would include: mitotic nondisjunction with loss of the wild-type chromosome (Fig. 1*c*) resulting in hemizygosity at all loci on chromosome 13, mitotic nondisjunction with reduplication of the mutant chromosome (Fig. 1*d*) resulting in homozygosity at all loci on the chromosome, or mitotic recombination (Fig. 1*e*) between the *Rb-1* locus and the centromere, resulting in heterozygosity at loci in the proximal region of the chromosome and homozygosity throughout the rest including the *Rb-1* locus. Several other more regionalized events such as gene conversion (Fig. 1*f*), deletion (Fig. 1*g*) or mutation (Fig. 1*h*) must also be considered. Both somatic and familial retinoblastoma could arise through the appearance of homozygosity at the *Rb-1* locus, the difference being two somatic events in the former and one germinal and one somatic event in the latter case.

Our approach to testing these hypotheses relies on the variability of DNA sequences among humans[11] which results in inherited differences in restriction endonuclease recognition sites. Recombinant DNA libraries[12,13] have been constructed from human–rodent hybrid cell lines which have segregated most human chromosomes other than chromosome 13. Segments of DNA which are homologous to unique loci on chromosome 13 and which reveal restriction fragment length polymorphism have been isolated, and their subregional localization determined[14–16]. Using these recombinant DNA segments as probes, we have examined loci scattered along the chromosome to detect changes in the somatic genotypes of tumours as compared with the germ-line genotypes of the individuals harbouring the tumours.

Mechanism of hemizygosity

The simplest way to reveal a recessive mutant allele at the *Rb-1* locus may be by loss of the corresponding homologous wild-type chromosome as depicted in Fig. 1*c*. This would result in hemizygosity at all loci on the remaining chromosome. If, in addition, the remaining chromosome carried a deletion of a specific section, then genes lying within the deleted portion of the chromosome would be completely absent from the tumour cells. Two of us (W.B., A.L.M.) recently reported a patient with sporadic retinoblastoma, LA-RB69A, whose normal constitutional cells exhibited half the esterase D activity shown by similar cells obtained from her parents, although the karyotype showed no detectable deletion of chromosome 13. An examination of her tumour cells revealed an absence of esterase D activity and karyotyping indicated that only one chromosome 13 was present. The interpretation of these results was that the patient carried a constitutional deletion of the *Rb-1* and *ES-D* loci which was invisible in the light microscope; furthermore, the tumour contained only the deleted chromosome 13[17]. However, the absence of the esterase D gene product in the tumour might still have been due to a specific somatic inactivation event, as the tumour cells also showed six unidentified marker chromosomes, any one of which might have included a portion of the wild-type chromosome 13. We tested these possibilities by

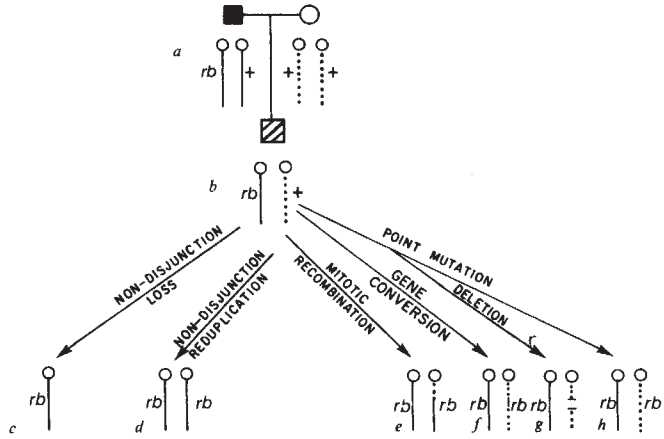

Fig. 1 Chromosomal mechanisms which could reveal recessive mutations. In this example, an affected male (*a*, ■) who carries a recessive defect at the *Rb-1* locus on chromosome 13, designated '*rb*', in all of his cells mates with a genotypically wild type, '+', female (*b*, ○). One of their children (Fig. 1*b*, ▨) inherits the defective chromosome 13 from his father and so is *rb*/+ at the *Rb-1* locus in all his cells. A tumour of his retinal cells may develop by eliminating the dominant wild-type allele at the *Rb-1* locus by the mechanisms required to effect the tumour cell genotype shown schematically in *c–h*.

examining the patient's constitutional and tumour cells for their respective genotypes at 7 loci on chromosome 13 defined by 10 restriction fragment length polymorphisms. As Fig. 2 shows, 2 of the 10 DNA markers were informative, that is, heterozygous in the constitutional tissue of the patient: p1E8 (2.1) which is homologous to a locus within q22–qter, and p7F12 (4.2) which is homologous to a locus within q12–q14 (ref. 12). At both loci, only one of the co-dominant alleles was evident in the tumour (Fig. 2*a*, *b*), supporting the original interpretation of chromosomal loss[17]. Figure 2*c* summarizes these data and suggests that the reduction to nullizygosity at the *Rb-1* locus in this tumour occurred by segregation of the wild-type chromosome.

Mechanisms of homozygosity

Figure 1*d* shows a variation on the nondisjunction mechanism in which, together with the nondisjunctional loss of the wild-type chromosome, the mutant chromosome is reduplicated. Such events would be difficult to detect karyologically or by quantitation of esterase D activity, but the use of co-dominant DNA markers should enable us to detect these events as a loss of one allele at each informative locus on the chromosome. A candidate for such an event has recently been presented[18] and is described in Fig. 3. This patient was heterozygous at the *ES-D* locus and showed no visible abnormality of either chromosomes 13. An examination of the tumour derived from this individual again showed no abnormalities of chromosome 13, except that two copies of the chromosome were present and another copy was present as a translocation involving chromosomes 13 and 14. However, the tumour cells exhibited only one of the two isozymic types of the esterase D enzyme, the allele from the father. It was proposed[18] that this resulted from somatic inactivation of the maternally derived allele of the *ES-D* locus on one homologue of chromosome 13.

Constitutional and tumour cells derived from this patient were tested with our seven recombinant DNA probes as described above. Three of the probes revealed heterozygosity in the constitutional tissue: p9A7 (0.95) which maps in the region q22–qter, and pHU26 and pHU10, both of which map in the region q12–q22. In each of these cases (Fig. 3*a–c*), although both co-dominant alleles were present constitutionally, only one allele at each locus was present in the tumour, and in each case this was derived from the paternally inherited chromosome 13 (data not shown). Our interpretation of these results is shown schematically in Fig. 3*d*. Rather than somatic inactivation of the

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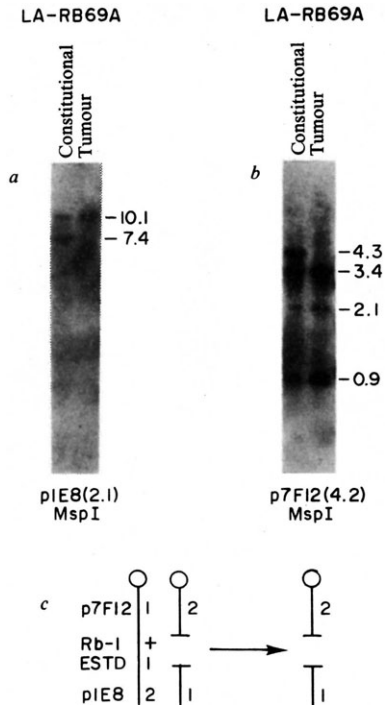

Fig. 2 Hemizygosity arising by segregation of one chromosome 13 homologue. *a*, The pattern obtained with hybridization probe p1E8(2.1) which contains a single-copy DNA insert homologous to a locus on human chromosome 13 which maps between band q22 and the terminus of the long arm[12]. *b*, The pattern obtained with hybridization probe p7F12 (4.2) which contains a single-copy DNA insert homologous to a locus on human chromosome 13 which maps between bands q12 and q14 (ref. 12). *c*, A schematic diagram incorporating data obtained by examination of the karyotype and esterase D alleles present in the two samples[17] with the data from *a* and *b*. Alleles at the loci homologous to the recombinant DNA probes are denoted 1 or 2 for the faster and slower alleles, respectively. The more common esterase D allele is designated 1, the less common 2 and the wild-type allele at the *Rb-1* locus is signified with a plus sign, while a mutant allele at this locus is denoted *rb*.

Methods: High molecular weight DNA was isolated from a lymphoblastoid cell line derived from peripheral lymphocytes and tumour cells which had been passaged twice intraocularly in nude mice, both from patient LA-RB69 (ref. 17). The DNA was digested to completion with *Msp*I, subjected to electrophoresis through 0.8% agarose gels, transferred to Zetapor membranes (AMF-Cuno) in 1.5 M NaCl/0.15 M Na-citrate, and hybridized to ^{32}P-labelled probes for 36 h. Filters were washed twice at room temperature in 0.3 M NaCl/0.03 M Na-citrate/0.1% SDS, once at 60 °C in the same solution, twice at 60 °C in 0.015 M NaCl/0.0015 M Na-citrate/0.1% SDS, and then exposed to Kodak XAR-5 X-ray film backed with Dupont lightning-plus intensifying screens for 1–3 days.

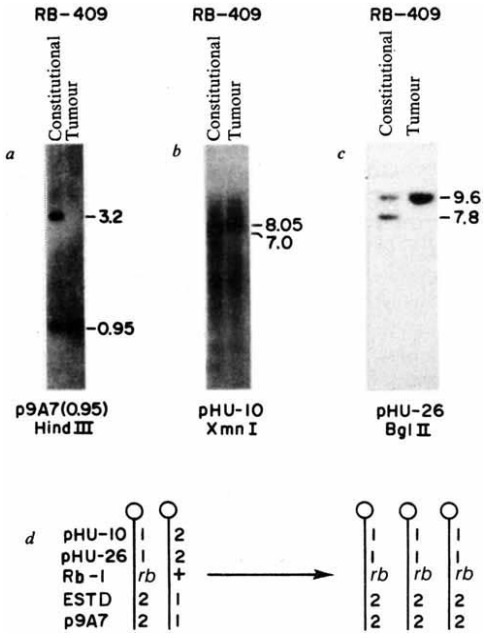

Fig. 3 Homozygosity effected by segregation of one chromosome 13 homologue with reduplication of the remaining one. *a*, Results obtained when *Hin*dIII-digested DNA was hybridized to p9A7 (0.95) which contains an insert homologous to a locus on chromosome 13 which maps between band q22 and the terminus of the long arm[12]. *b*, The pattern obtained when *Xmn*I-digested DNA was hybridized to the insert fragment derived from the plasmid pHU10 which is homologous to a locus on chromosome 13 which maps between bands q12 and q22 (ref. 13). *c*, The pattern obtained when *Bgl*II-digested DNA was hybridized to the insert fragment isolated from the plasmid pHU26 which is homologous to a locus on chromosome 13 which maps between bands q12 and q22 (ref. 13). *d*, A schematic diagram incorporating these data with previous analyses[18] of the esterase D alleles present and the karyotype of the two samples from patient Rb-409.

Methods: High molecular weight DNA was isolated from fibroblasts or tumour cells maintained as primary tissue cultures, both from patient Rb-409 (ref. 18). The DNA was digested to completion with the indicated restriction endonucleases, resultant fragments separated by electrophoresis in 0.8% agarose gels, transferred to Zetapor (*a*, *b*) or nitrocellulose (*c*), hybridized to ^{32}P-labelled probes, washed and exposed to X-ray film as described in Fig. 2 legend.

ES-D locus on one chromosome13 homologue, these data are consistent with the complete loss of the entire maternally derived chromosome, with reduplication of the paternally-derived chromosome. We assume that this chromosome carried a *de novo* germinal mutation, as the father showed no evidence of retinoblastoma, but the individual was bilaterally affected. It is also possible that one or two of the chromosomes 13 in the tumour were derived by a mitotic exchange between the wild-type and mutant chromosomes (Fig. 1*e*) such that an original mutant chromosome and a recombinant chromosome (or two) are maintained. If this had happened, the point of interchange must lie proximal to the region detected by the most proximal marker locus, since all the markers including *ES-D* (which maps to q14) are homozygous in the tumour.

Figure 1*e* shows another possible mechanism whereby part of a pair of chromosomes may become homozygous, although not previously observed in man. A somatic, or mitotic, recombination between the mutant and wild-type chromosome homologues, with subsequent segregation, can result in a cell which maintains heterozygosity at loci proximal to the breakpoint of the recombination but which shows homozygosity at loci distal to such a breakpoint. Examination of an instance of such a mechanism in patient Rb-412 (ref. 18) revealed that the constitutional cells from this case of apparent sporadic retinoblastoma were heterozygous both at the *ES-D* locus as well as for a quinacrine-staining satellite heteromorphism on the short arm of the chromosomes 13[18]. The tumour cells from this patient showed the presence of both types of satellite staining, but only one isozymic form of esterase D. A reasonable interpretation of these data[18] is that both chromosomes 13 are present, in their entirety, in the Rb-412 tumour and that a somatic inactivation of the *ES-D* locus on one homologue results in hemizygosity and subsequent detection of only one isozymic form of the enzyme. Alternatively, a mitotic recombination event occurring between the centromere and the *ES-D* locus, as described above, could also generate chromosomes consistent with these results. Figure 4 shows the results obtained when constitutional and tumour genotypes of this patient were examined at chromosome 13 loci defined by seven DNA probes. Three of the markers were heterozygous in skin fibroblasts from Rb-412: p1E8 (2.1) which maps distal to q22; p9D11 (2.3) which maps at q22; and p7F12 (4.2) which maps between q12 and q14. Both p1E8 (2.1)

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(Fig. 4*a*) and p9D11 (2.3) (Fig. 4*b*) reduced to homozygosity in the tumour tissue. However, p7F12 (4.2), the marker locus which maps between the *Rb*-1 locus and the centromere, retained heterozygosity (Fig. 4*c*). Our interpretation of these results, taken together with the heterochromatic staining and esterase D data described above, is illustrated in Fig. 4*d*. We suggest that recombination occurred between the mutant and wild-type chromosomes 13 in the cell that gave rise to the tumour. The cross-over point was between the 7*F*12 locus and the *Rb*-1 and *ES-D* loci such that all loci distal to the cross-over point, including *Rb*-1 have become homozygous. Between *Rb*-1 and the terminus of the short arm, however, two loci maintain both the maternal and paternal haplotypes. This is, to our knowledge, the first characterization of *in vivo* mitotic recombination in mammals. We do not know which chromosome carried the original mutation (that is, displayed no heterochromatic staining in the diagram) nor which chromosomal haplotype was associated with which satellite heteromorphism. However, we are investigating these questions directly by examining the haplotypes present in human–rodent hybrid cells segregating the two chromosomes 13.

Other retinoblastoma genotypes

The same comparison of tumour and constitutional genotypes as described above were performed in five sporadic additional cases[18], and the results are summarized in Table 1. One case, Rb-430, demonstrated a loss of one esterase D isozymic form in the tumour. Only one of the DNA probes (pHUB8) showed heterozygosity in fibroblasts from that patient and it was reduced to homozygosity in the tumour. As the locus homologous to this probe maps between q12 and q22, as does esterase D (q14), we cannot tell which, if any, of the mechanisms outlined in Fig. 1*c–h* pertain to this case. However, the lack of visible chromosome abnormalities makes less likely both nondisjunctional loss and a large deletion, whereas the reduction to homozygosity at two loci makes point mutation and, unless the two loci are very close together, limited gene conversion less likely also. Thus, the more likely possibilities for this tumour are nondisjunction with reduplication or mitotic recombination similar to either Rb-409 (Fig. 3) or Rb-412 (Fig. 4), respectively. Note that the RB-430 tumour material examined was obtained directly from the patient without growth in culture or in nude mice.

Another case, Rb-267, was uninformative for esterase D, but one marker, p7F12 (4.2), mapped to q12–q14, became homozygous in the tumour. As the karyotype of these tumour cells showed two chromosomes 13, neither of which seemed to have a deletion, nondisjunction with chromosome loss or deletion is unlikely. Unless a second mutation as shown in Fig. 1*h* occurred in the polymorphic *Taq*I recognition site, or a limited gene conversion event (Fig. 1*f*) included the site, these mechanisms are also unlikely. Thus, either nondisjunction with chromosome reduplication or mitotic recombination seem most probable. Thus, with these two cases, and the three cases described in Figs 2–4, we have observed a reduction to homozygosity or hemizygosity at at least one locus on chromosome 13 in five separate tumours.

However, the analyses of tumours Rb-247, Rb-405 and Rb-355 (Table 1) indicate that these rather gross chromosomal mechanisms are not universally applicable, as heterozygosity was maintained in the tumours at three (Rb-247 and Rb-355) or four marker loci (Rb-405). Clearly, neither nondisjunction with chromosome loss and reduplication nor mitotic recombination occurred in these cases. Large deletions are also excluded by microscopic evidence. Thus, we suggest that the secondary event revealing the initial recessive mutation would itself be either a point mutation or a small deletion at the homologus *Rb*-1 locus (Fig. 1*h*) or a regionalized recombinational event such as gene conversion (Fig. 1*f*).

Specificity to chromosome 13

Because we have examined loci only on chromosome 13 in these tumours, we were unable to evaluate the chromosome specificity

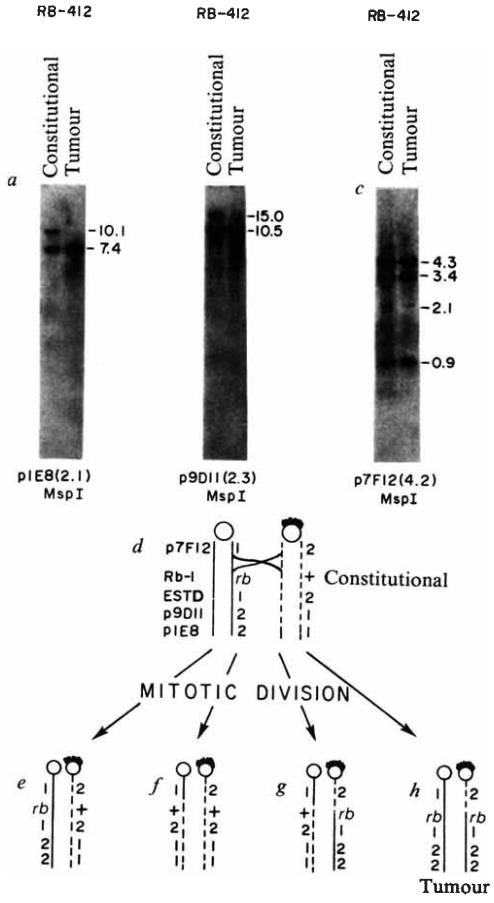

Fig. 4 Homozygosity effected by a mitotic recombinational event. *a*, The pattern obtained when the hybridization probe was p1E8 (2.1) which is homologous to a locus on chromosome 13 mapping between band q22 and the terminus of the long arm[12]. *b*, Results obtained when the hybridization probe was p9D11 (2.3) which is homologous to a locus on chromosome 13 mapping to band q22 (ref. 12). *c*, Results obtained when the hybridization probe was p7F12 (4.2) which is homologous to a locus on chromosome 13 mapping between bands q12 and q14 (ref. 12). *d*, Schematic diagram showing our inferred haplotypes on each chromosome derived from karyology, esterase D determinations[18] and data described here. The cap on the chromosome homologue represented by the dashed lines denotes a fluorescent-staining heterochromatin region[18]. In this figure the point of cross-over must lie between the *Rb-1* and *7F12* loci, and is shown occurring between chromatids of the chromosome 13 homologue at the four-strand stage of mitotic chromosome replication. *e–h*, Schematic diagram of the four possible combinations of wild-type and recombinant homologues. Possibilities *e–g* result in a phenotypically wild-type cell and are inconsistent with the allelic data shown in *a–c*, whereas the combination shown in *h* corresponds to the experimental data and results in homozygosity for the mutant (*rb*) allele at the *Rb-1* locus.

Methods: High molecular weight DNA was isolated from fibroblasts and tumour cells maintained as primary tissue cultures, both from patient Rb-412 (ref. 18). The DNA was digested to completion with *Msp*I, the resultant fragments were separated by electrophoresis through a 0.8% agarose gel, transferred to Zetapor membranes, hybridized to ^{32}P-labelled probes, washed and exposed to X-ray film as described in Fig. 2 legend.

of the development of homozygosity observed. A recent survey[19] of many different types of tumour suggests that the frequency of heterozygosity for several polymorphic enzyme markers is lower than would be expected based on the allele frequencies at such loci in the general population. That study did not, however, compare isozyme patterns in tumour and constitutional tissues from the same patient. To examine the specificity of the changes, we examined the same Southern transfers of DNA isolated from tumour and normal tissues

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Table 1 Alleles at chromosome 13 loci in other cases

		Alleles present in									
		RB267		RB430		RB247		RB405		RB355	
Probe	Enzyme	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour
p1E8	*Msp*I	2, 2	2, 2	1, 1	1, 1	1, 2	1, 2	1, 2	1, 2	1, 1	1, 1
p7F12	*Msp*I	1, 2	2, 2	1, 1	1, 1	1, 1	1, 1	1, 2	1, 2	1, 2	1, 2
	*Taq*I	1, 2	2, 2	1, 1	1, 1	1, 1	1, 1	2, 2	2, 2	1, 2	1, 2
p9A7	*Msp*I	2, 2	2, 2	2, 2	2, 2	1, 1	1, 1	1, 1	1, 1	1, 2	1, 2
p9D11	*Msp*I	2, 2	2, 2	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1
	*Taq*I	2, 2	2, 2	2, 2	2, 2	2, 2	2, 2	2, 2	2, 2	2, 2	2, 2
pHU-B8	*Eco*RI	1, 1	1, 1	1, 2	1, 1	1, 1	1, 1	1, 1	1, 1	1, 2	1, 2
	*Hin*dIII	1, 1	1, 1	1, 1	1, 1	1, 2	1, 2	1, 1	1, 1	1, 1	1, 1
pHU-10	*Xmn*I	1, 1	1, 1	1, 1	1, 1	1, 2	1, 2	1, 2	1, 2	1, 1	1, 1
pHU-26	*Bgl*II	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1	1, 1
Esterase D		1, 1	1, 1	1, 2	1, 1	1, 1	1, 1	1, 2	1, 2	1, 2	1, 2

DNA was isolated, digested, transferred to Zetapor membranes, hybridized to the indicated ^{32}P-labelled probes, washed and exposed to X-ray film as described in Fig. 2 legend. Tumour tissue was from primary tissue cultures except in RB-430 in which the tumour tissue was obtained directly from the patient. Esterase D data and karyotypic analysis have been reported previously[18].

Table 2 Alleles present at loci on chromosomes other than 13

			Alleles present in							
			RB-409		LA-RB-69A		RB-267		RB-355	
Probe	Enzyme	Chromosome	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour
DOSCL-2	*Msp*I	20	1, 2	1, 2						
DOSLC-3	*Msp*I	15	1, 2	1, 2	1, 2	1, 2				
DOSLC-4	*Msp*I	17	1, 2	1, 2	1, 2	1, 2				
DOSLC-9	*Msp*I	3			1, 2	1, 2	1, 2	1, 2	1, 2	1, 2
DOSLC-6	*Taq*I	18					1, 2	1, 2		
DOSLC-8	*Taq*I	11			2, 3	2, 3	1, 3	1, 3	2, 3	2, 3
KRAS2	*Taq*I	12					1, 2	1, 2	1, 2	1, 2
HRAS1	*Taq*I	11	1, 2	1, 2	1, 2	1, 2				

			RB-247		RB-405		RB-430		RB-412	
Probe	Enzyme	Chromosome	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour	Constitutional	Tumour
DOSLC-2	*Msp*I	20	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2
DOSLC-3	*Msp*I	15	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2		
DOSLC-4	*Msp*I	17							1, 2	1, 2
DOSLC-9	*Msp*I	3	1, 2	1, 2	1, 2	1, 2			1, 2	1, 2
DOSLC-6	*Taq*I	18	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2
DOSLC-8	*Taq*I	11	1, 3	1, 3			1, 3	1, 3	2, 3	2, 3
KRAS2	*Tqa*I	12	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2	1, 2
HRAS1	*Taq*I	11							1, 2	1, 2

The same Southern transfers of tumour and constitutional DNA described in Figs 2–4 and Table 1 legends were hybridized to the indicated ^{32}P-labelled probes. Each of the probes was previously mapped to the chromosome shown and is indicated by the standard nomenclature assigned by the International Workshop on Human Gene Mapping[28]. Only those instances in which heterozygosity was present in the constitutional cells of each case are reported here; all blanks represent instances in which homozygosity was observed.

described above with eight DNA segments homologus to loci on seven different chromosomes (Table 2). In all cases in which the patient was heterozygous constitutionally, the corresponding loci in the tumour were also heterozygous. In all cases where we observed reductions to homozygosity at loci on chromosome 13 (Rb-267, Rb-430, Rb-412, Rb-409, LA-RB69A), markers on four to six other chromozomes maintained heterozygosity in the tumours. Thus, we suggest that although these chromosomal mechanisms may be general, albeit at low frequency, only those events resulting in homozygosity at the *Rb*-1 locus on chromosome 13 cause a retinoblastoma tumour.

Discussion

We have described here a new approach to the somatic cell genetics of tumorigenesis *in vivo*. Our results bring together the hypothesis that neoplasms can arise in a two-step manner[6–10] with the suggestion that chromosomal events can lead to tumour formation[17,20,21] and specifically with evidence that chromosome loss[22] or loss and reduplication[23] can lead to the expression of recessive mutations[22]. These events can be such that the absence of a chromosome would be detected karyologically, as with LA-RB69A. However, in at least two other cases (Rb-409 and Rb-412), these events would go unseen by conventional high resolution microscopic examination, that is, we have also described an approach to karyology at the molecular level. Note also that in the tumour tissue we examined, all observed reductions to homozygosity seemed to be complete. The observation that the change was qualitative rather than quantitative suggests a clonal origin of the tumours.

The recombinant DNA libraries of chromosome 13, from which we isolated the polymorphic DNA markers used in these studies, will yield many more such markers. Subsequent examinations of tumour and constitutional genotypes may allow us to search for small recombination or gene conversion (Fig. 1*f*) events within specific small segments of this chromosome. Confirmation of the occurrence of a single point mutation at the *Rb*-1 locus will require the isolation of the gene whose defect predisposes to retinoblastoma and molecular analysis of the wild-type and mutant forms of that gene. However, such a gene is unlikely to be defined in an interspecies transfection assay such as that described for other oncogenes[24–26] because this

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assay would require that the donor gene be expressed in a dominant fashion, whereas the present data suggest that mutant retinoblastoma alleles are recessive and will, therefore, not be revealed by these operational definitions. Thus, a new approach must be devised which allows the assay and isolation of recessive oncogenes.

Although our evidence suggests that retinoblastoma can result from the development of homozygosity for recessive mutant alleles at the *Rb-1* locus, additional dominant mutations may also be involved. Patients having the heritable form of retinoblastoma have a high probability of developing other cancers, especially osteogenic or fibrosarcomas[27]. We have not examined the constitutional and tumour genotypes in such cases, but we may observe reductions to homozygosity at loci on chromsome 13 with or without an additional dominant mutation. This secondary tumour material could serve as a useful source of DNA for transfection assays which might expose steps in the pathway in addition to those described here.

Furthermore, although the present experiments support the hypothesis that the chromosome remaining in the tumour cells carries the *rb* mutation (or a recombinant thereof), in only one case, LA-RB69A, is there direct evidence of this. In this tumour, the absence of esterase D activity[17] indicates that it is the mutant chromosome which remains. Further evidence for this must await the direct identification of the afflicted chromosome in those inherited cases where the genotype of an affected parent can be determined. These materials have been difficult to obtain due to early detection in such genetically predisposed individuals and their subsequent early treatment with radiation or chemotherapy.

We further suggest that chromosomal nondisjunction and mitotic recombination may be involved in the development of other tumours in addition to retinoblastoma. The observation of development of homozygosity using the approach described here can be applied to many other tumour types by using a battery of probes for loci which span the genome. A positive result with such a screen would confirm the role of this mechanism in the genesis of other tumours and may lead to chromosomal localization of the gene(s) of interest. Finally, if this mechanism of carcinogenesis is generalized to other, more common tumours, it will become important to test environmental agents for their activity in stimulating nondisjunction and mitotic recombination events, in addition to their mutagenic activities, in order to determine their carcinogenic potential.

The probe homologous to the c-Ha-*ras*2 locus on chromosome 11, pTBB-2, was a gift from Mike Wigler. The probe homologous to the c-Ki-*ras*2 locus on chromosome 12, p640, was kindly supplied by Bob Weinberg. We thank Becky Cornwall for her help in the preparation of the manuscript. These studies were supported by the Howard Hughes Medical Institute and, in part, by grants from the NIH, the Retina Research Foundation, the NCI of Canada, the Hospital for Sick Children Foundation, the MRC of Canada, and in conjunction with the Clayton Foundation for Research.

Received 27 June; accepted 8 August 1983.

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LETTERS TO NATURE

Observation of γ rays $>10^{15}$ eV from Cygnus X-3

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The X-ray binary system Cygnus X-3 is a source of particular interest. As well as emitting X rays which are modulated with a 4.8-h period, it has been observed in 30–100 MeV γ rays by the SAS II satellite[1] and several groups have detected γ rays in the TeV range showing the same 4.8-h period[2–5]. Additionally there is evidence of a 34.1-day period[2,6,7] and of random variations correlated with X-ray emission and radio outbursts[8,9]. Most recently, Samorski and Stamm[10], using the small extensive air shower array at Kiel, have found that the γ-ray spectrum of Cygnus X-3 extends above 2×10^{15} eV, with an integral flux of $(7.4\pm3.2)\times10^{-14}$ cm^{-2} s^{-1}. This result has considerable implications both for our understanding of the source and for the solution of the long-standing problem of the origin of cosmic rays. We now confirm the Kiel observations and present evidence that the γ-ray spectrum of Cygnus X-3 steepens above 10^{16} eV.

The results reported are based mainly on measurements with a small shower array which comprises four detectors; three lie on the circumference of a circle of 50 m radius with the fourth at the centre of the circle. The detectors are each 13.5 m$^2\times$1.2 m deep water-Čerenkov detectors. This array (B), which is sensitive to the range 10^{15}–10^{16} eV, is part of the giant Haverah Park array[11] and is located at latitude 53.9° N and altitude 198 m. An investigation of the decade above 10^{16} eV was made using two larger arrays (E, G) of identical geometry but with a radius of 150 m. Conventional techniques are used to measure the incident direction of each air-shower and to determine a parameter which can be used to infer the primary energy of each event. Details of these procedures will be given elsewhere.

Showers were selected with zenith angles, $\theta<30°$ (array B) or $\theta<40°$ (arrays E, G) and core distances from the central detector $\leq$ the array radius. Primary particle directions for this subset can be measured with an uncertainty of $\sim10^{-2}$ sr. Higher precision is not possible because of the large size of the Čerenkov detectors, the risetime of the electronics (including the photomultipliers, EMI 9618YB) and the uncertainty in correcting for shower front curvature. In view of the estimated angular uncertainty, the data from a region of 9° in right ascension and 6° in declination centred on Cygnus X-3 ($\alpha=307.8°$, $\delta=40.9°$) have been examined and compared with the 'off-source' sky area in the same 6° declination strip, resulting in the count rates given

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Table 1 Summary of Observations on Cygnus X-3

	Array B(50 m) $E > 10^{15}$ eV	Arrays E, G(150 m) $E > 10^{16}$ eV
Data period (JD−2440000)	3,874–5,335	3,509–5,335
On-time efficiency %	89	81
Time 'on-source' (hours)	8,945	27,767
'On-source' count	1,627 ± 40	397 ± 20
'Off-source' count	1,559 ± 6.3	424 ± 3.3

in Table 1. For the data from array B, the source region shows an intensity only slightly above (1.7 σ) the background intensity, which can be determined with high accuracy from the remaining 351° RA of the off-source region. In view of the intensities reported by the Kiel group[10], and our angular resolution, the lack of a significant excess in the time-averaged data is not unexpected.

To identify a periodicity in a data stream extending over more than 4 y requires accurate knowledge of the source ephemeris. The consensus view of those using the air-Čerenkov technique in the TeV range is that the ephemeris of van der Klis and Bonnet-Bidaud[12] should be adopted. We have applied this ephemeris to the 'on-source' events of Table 1 after correcting each arrival time to the heliocentric coordinate system; a correction to barycentric arrival time is not warranted as the event arrival time resolution is 30 s.

Figure 1*a* shows the resulting phase histogram for the lower energy data (array B), binned into 40 intervals to accommodate the uncertainty in extrapolating the ephemeris. The peak in the phase interval 0.225–0.25 (relative to X-ray minimum) is significant: the poissonian probability of observing any one of 40 bins with an event count of $\geqslant 73$ when 39.0 are expected is 2.8×10^{-5}, and a χ^2 test on the phase histogram uniformity about the off-source mean yields $\chi^2_{40} = 1.78$, $(P(>\chi^2) = 1.8 \times 10^{-3})$. The relative likelihood method[13] rejects the hypothesis of uniformity in phase, H_0, in favour of the hypothesis, H_1, that there is an enhanced flux in any one of the 40 phase bins, at a confidence level of 99.97%. It is quite possible that all of the time-averaged excess is contained within this single interval (pulsed excess/time-averaged excess = 50 ± 30%) since the phase histogram without this interval is consistent with noise ($\chi^2_{39} = 1.07$). There is no evidence from arrays E and G for nonuniformity in phase above 10^{16} eV: the relative likelihood ratio for H_1 against H_0 is 1.01.

To test further the genuine nature of the pulsed emission above 10^{15} eV we have examined the off-source data, similarly folded with the ephemeris: the phase histogram is consistent with noise ($\chi^2_{39} = 0.87$). A crucial test is to examine all events recorded in the same zenith angle band as Cygnus X-3, but at off-source azimuth angles. Such a test is sensitive to any nonuniformities in phase detection efficiency, which may be caused by on-time or atmospheric pressure variations (for array B the rate of shower detection changes by -1.0% mb^{-1}): again the phase distribution is consistent with noise ($\chi^2_{39} = 1.08$). For comparison with Fig. 1*a* we show, in Fig. 1*b*, the product of the phase detection efficiency, due to on-time and atmospheric pressure variations, with the off-source mean; clearly the contribution from these variations is well below the statistical noise.

The inferred width of the γ-ray pulse (and hence its statistical significance) is very sensitive to the adopted ephemeris because of the necessity of extrapolating the ephemeris parameters (the period p and its derivative $\dot{p}$) over more than 4 y. For illustration, our data are shown in Fig. 1*c*, *d*, folded with the ephemerides of Parsignault *et al.*[14] and Elsner *et al.*[15] respectively. The Kiel group, using the former ephemeris extrapolated by ~120 days, obtained a significant excess in the phase interval 0.35–0.4. Our observation period requires an extrapolation of ~1,140 days

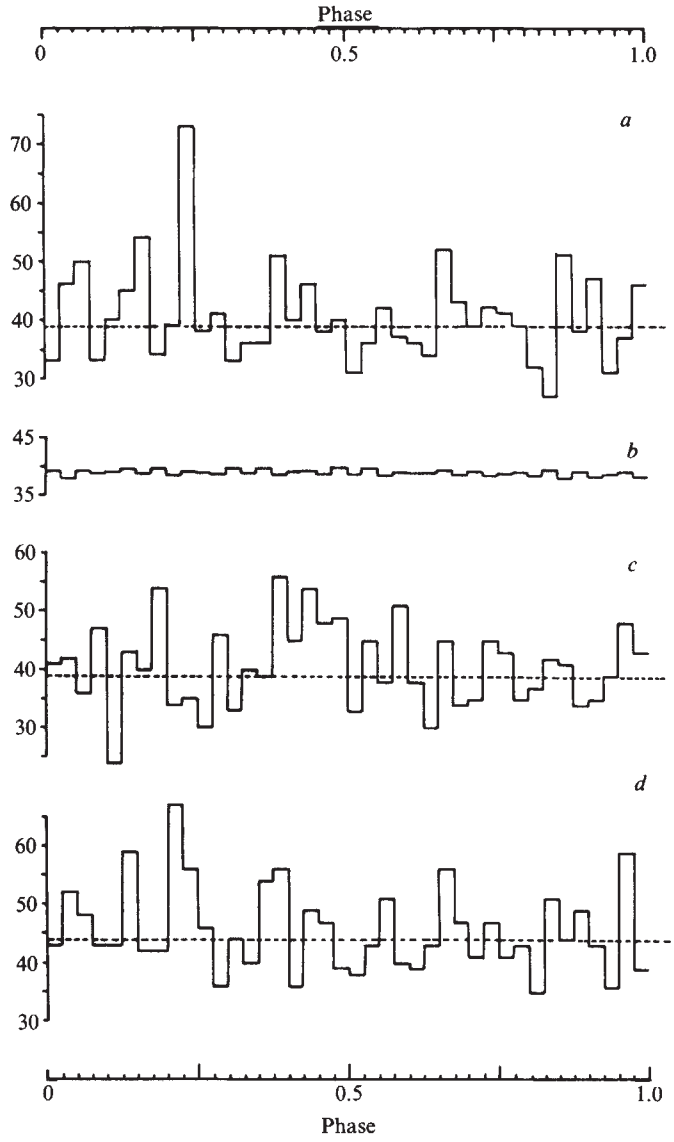

Fig. 1 Phase distributions of events from array B within 9° × 6° of Cygnus X-3, folded with a 4.8-h period relative to X-ray minimum. In *a*, *c*, *d* the data are folded using the ephemerides of refs 12, 14, 15 respectively; the dotted lines represent the mean count 'off-source'. The product of the detection efficiency and 'off-source' mean is plotted in *b*, showing the expected contribution to phase nonuniformity from both on-time and atmospheric pressure variations.

for that ephemeris, and it is apparent from Fig. 1*c* that this results in loss of significance.

Parsignault *et al.* restricted the fitting of their observations to the phase interval 0.6–1.0 to avoid the large intensity fluctuations observed at earlier phases. This results in a systematic phase difference (~+0.2) between their ephemeris and those of others[12,15]; there is some evidence for this difference in the phase histograms of Fig. 1*c*, *d*. The ephemeris of Elsner *et al.* is derived from a compilation of many measurements spanning 8 y, the observations ending 16 days before the start of our observations. We note that it is only the most contemporary ephemeris[12] which provides a strongly significant excess in a very narrow interval of phase, and therefore we regard this as the most suitable for extrapolation.

Figure 2 compares the present results on the phase of the pulsed emission with previous results above 500 GeV. The present measurement, and that of the Kiel group, strongly indicates that above 10^{15} eV this is narrowly confined to the phase region near 0.25. Note that an average adjustment (−0.11) has been made to the phase reported by the Kiel group to comply with

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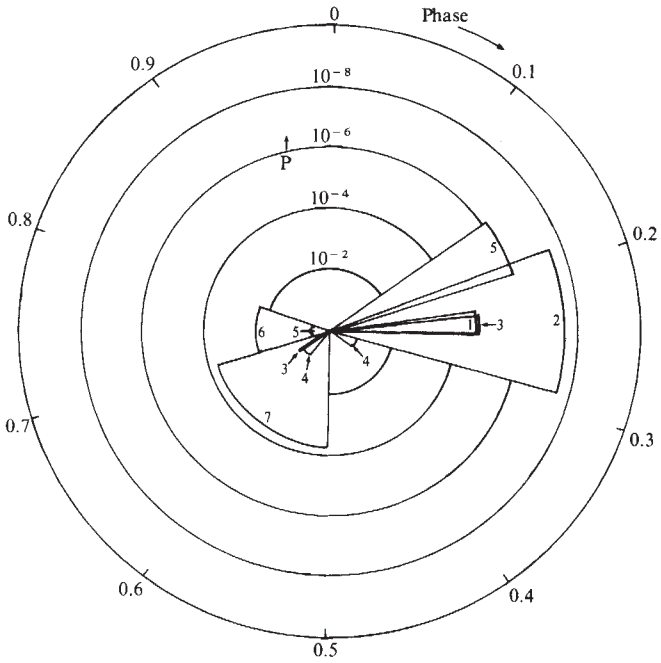

Fig. 2 A summary of the phases (relative to X-ray minimum) for pulsed emission above 500 GeV from Cygnus X-3. The numbered sectors, 1–7, are taken from the present work and refs 10, 10, 18, 2, 3, 4 respectively. The probability of each measurement occurring by chance has been estimated from the cited references (where not explicitly stated) and is plotted radially.

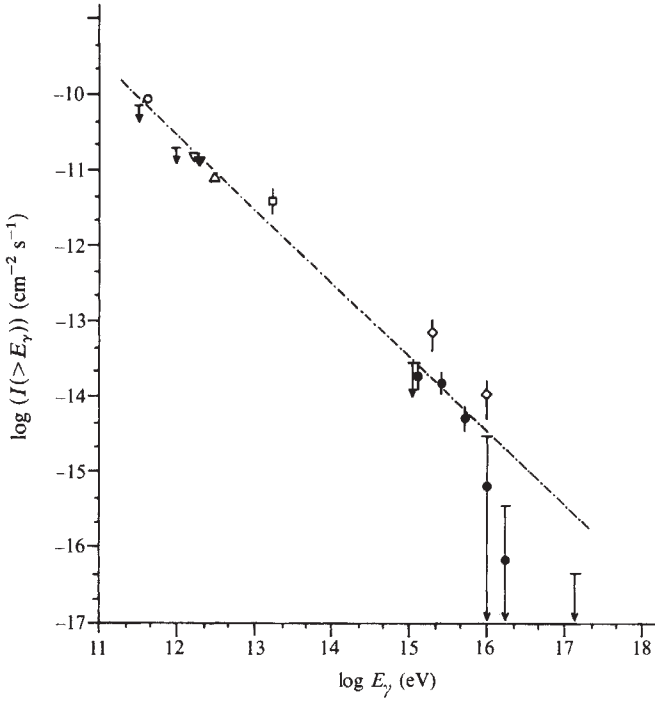

Fig. 3 The time-averaged integral γ-ray flux above 10^{11} eV from Cygnus X-3. Source of measurements: ○, ref. 4; ▽, ref. 2; ▼, ref. 3; △, ref. 5; □, ref. 18; ◇, ref. 10 and ●, this work. The upper limits are obtained from refs 19 and 20 below 10^{12} eV and at 10^{15} eV respectively, and the present work (95% limits) above 10^{16} eV. The dotted line is an estimate of the spectral slope between 5×10^{11} eV and 5×10^{15} eV.

our adopted ephemeris (the adjustment required by the other ephemerides is <0.05). Only one other experiment[2], at lower energy, has reported a significant excess with similar phase; the remaining results, in the TeV region, consistently show an excess in the region 0.6–0.7.

In view of earlier reports of time variability of the Cygnus X-3 signal at TeV energies and below, we have divided our data from the phase bin 0.225–0.25 into yearly intervals. Maximum likelihood estimates[13] of the excess counts in this bin for each year, 1979–82, are $10.4^{+4.8}_{-4.2}$, $14.3^{+5.3}_{-4.6}$, $7.4^{+4.5}_{-3.8}$, and ≤ 10.7 (95% limit) respectively. The absence of a signal in 1982 may reflect a statistical fluctuation, genuine time variability or failure of the ephemeris extrapolation. We plan to use an ephemeris based on recent EXOSAT observations (D. Andrews and A. Smith, personal communication). However, analysis with a preliminary updated ephemeris using COS B X-ray observations in November 1981 and February 1982 (M. van der Klis, personal communication) does not improve the significance of the signal in 1982.

The data of 1979 and 1980, in the phase interval 0.225–0.25, have been scanned for evidence of time variability on the scales of 10 days and 34.1 days. Five events were observed between JD2444032 and JD2444041 when 0.7 were expected; the probability of observing such an excess, after making due allowance for the scanning method[16], is 15%. The data, folded modulo 34.1 days, show no evidence for periodicity by a Rayleigh test (amplitude <0.78 with 95% confidence). In the 40- and 30-day periods following the radio outbursts of September 1980 and September 1982 respectively we observed 52 and 33 on-source events while 47 and 37 were expected.

Table 2 lists, in differential energy bins, the number of cosmic rays observed in the phase interval 0.225–0.25, compared with the numbers expected from the remainder of the period. From these data we have derived best estimates for the integral, time-averaged, γ-ray flux using the relative likelihood technique. The results are compared with other measurements above 500 GeV in Fig. 3. The energy assignments are derived assuming that (1) the lateral structure of γ-ray initiated showers is not markedly different from that of nuclei-initiated cosmic ray showers and (2) the experimental parameter which is used to determine primary energy is similarly insensitive to the initiating primary. These assumptions are in line with recent calculations (A. M. Hillas, personal communication) which show that both the depth of maximum and sea-level sizes of proton and photon-initiated showers differ by less than 30% at 10^{15} eV. The review spectrum of Hillas[17] has been used to assign the γ-ray and background cosmic ray energies.

Table 2 Observed and expected event counts for the phase interval 0.225–0.25 in differential energy bins

	Array B				Arrays E, G			
Energy (10^{15} eV)	1–3	3–5	5–11	11–21	18–36	36–72	72–140	>140
Observed	16	34	17	4	3	6	2	0
Expected	13.9	16.4	6.2	2.4	4.3	3.4	0.8	0.6

Systematic uncertainties in the energy assignments of all the data in Fig. 3 preclude a rigorous determination of the spectrum slope. Using the measurements[2–5] near 10^{11} eV and our own intensities at $1–5\times10^{15}$ eV, we have approximated the spectrum by a power law. The slope is flat, $\gamma\sim1.0$, and extrapolation to higher energies suggests that there is a cutoff in the γ-ray spectrum; above 2×10^{16} and 1.4×10^{17} eV, 52 and 16 events are predicted whereas the 95% upper limits from our measurements are 9.6 and 3 respectively. Inclusion of the remaining data of Fig. 3[10,18,19] can only strengthen this conclusion. Additionally the source spectrum near 10^{15} eV must be flatter than the observed spectrum because of attenuation by pair production reactions[21,22] with the microwave background. The attenuation length at 3×10^{15} eV is 7 kpc so that for a source distance of 10 kpc[23] the flux has been attenuated by ~4 whereas at 5×10^{16} eV the attenuation length is 50 kpc.

The integral intensity above 3×10^{15} eV is $(1.5\pm0.3)\times10^{-14}$ photons cm^{-2} s^{-1}. For isotropic emission and a source distance of 10 kpc, this implies a luminosity at $3–10\times10^{15}$ eV (corrected for γ–γ attenuation) of 3×10^{36} erg s^{-1}. We have no direct evidence that our signal is due to γ rays but the neutron primaries (decay length ~30 pc) imply a vastly enhanced luminosity.

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These results have implications for the pulsar-binary system model[24] of Cygnus X-3. In this model a pulsar, which can accelerate charged particles to high energies, orbits a companion star with a period of 4.8 h. The γ rays are supposed to arise from interactions of charged particles (electrons or protons) in the atmosphere of the companion. The work of Samorski and Stamm[10], and our own work, establish the existence of γ rays with $E > 5\times10^{14}$ eV, so that the origin of the γ rays as bremsstrahlung from shock-accelerated electrons appears to be excluded. We note that the lifetime of a 10^{16} eV electron is limited by synchrotron radiation losses to 25 ms in a 1 G field so that the progenitors of the γ rays may be π^0 particles produced by proton interactions in the atmosphere of the companion object. A cutoff in the γ-ray spectrum may therefore reflect the maximum proton energy reached in the pulsar accelerator.

We thank D. Andrews, T. L. Evans, A. M. Hillas, M. van der Klis, G. Navarra, N. A. Porter and K. A. Pounds for useful discussions and SERC for support of the Haverah Park project and for the provision of studentships to R.N.C. and A.L.

Received 27 July; accepted 22 August 1983.

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Can quarked niobium be a fission product?

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One description of nuclei[1–3] involves low-energy ground states of quark configuration $u^{2Z+N}d^{Z+2N}$ with atomic weights close to $(Z+N)=A$. If quarks are not unconditionally confined in their baryons, states with quark numbers not divisible by 3 have much higher (but definite) rest masses and carry electric charges $(Z\pm\frac{1}{3})e$. Evidence for unsaturated quarks in niobium[4] suggests two kinds of carriers of fractional charge, a positive species loosely bound to electrons[5,6] and a nucleus permanently bound to a negative quark[7,8]. However, it is argued here that the latter systems rather may contain $3A-1$ quarks, and may be formed primordially from a free anti-quark and a nucleus provided by the vacuum (simultaneously with an anti-nucleus of the same A). Whereas the ratio R between binding energy and rest mass of the products formed is 0.008 for most nuclei relative to nucleons, it is probably well above 50 for $3A$ quarks forming a nucleus, since no unsaturated quarks[9] were found (in positron–electron collisions) below 15 AMU (1 AMU = 0.9315 GeV).

If the dew-point (T) of quark condensation to nucleons[2] is $\sim2\times10^{12}$ K ($kT=0.2$ GeV) some 10^{-5}s after the big bang[10], one would expect[11] a surviving concentration of 10^{-20}–10^{-30} unsaturated quarks per nucleon, to be compared with one carrier of fractional charge[4] per 10^{18} niobium nuclei or 10^{20} nucleons, whereas negative results are reported[12] for 10^{20} nucleons in mercury. A suspicion is growing[1,13–16] that diquarks may be much lighter than a single quark, and perhaps have moderate masses around 20–50 AMU. The quark wavefunction of a nucleus clearly shows pronounced tendency of clustering to u^2d (protons), ud^2 (neutrons) and u^6d^6 (α-particles) in small volume elements on an instantaneous picture (compared with the much milder effects of correlation in electronic wavefunctions) but pions[3] and other instantaneous 'constituents' having typical hadronic radii are another model of displacing a quark between two adjacent nucleons. It is also clear that classifying sodium or thulium nuclei as $u^{34}d^{35}$ or $u^{238}d^{269}$ is like describing a solidified (or liquefied) noble gas by delocalized molecular orbitals. However, adding or removing one quark produces characteristics like a molecule with unpaired spin[17]. The concomitant properties are then further removed from those of a nucleon Hartree product wavefunction.

Once it is accepted that the cost, 1.88 GeV, of materializing a nucleon and an anti-nucleon from the vacuum is small, compared with the rest masses of systems involving unsaturated quarks, Umlagerung (switch) reactions[1] are feasible:

$$\begin{aligned}\bar{u}+[u^2d+(\bar{u})^2\bar{d}]&\rightarrow(\bar{u})^2\bar{d}+ud\\ d+[u^2d+(\bar{u})^2\bar{d}]&\rightarrow u^2d+(\bar{u})^2\end{aligned}\qquad(1)$$

The diquark ud charged $+e/3$ is a good candidate for the mobile charge carriers in niobium[5,6]. This would also be true for u^2 formed from $\bar{d}$ and vacuum, ejecting an anti-proton. The anti-diquark $(\bar{u})^2$ with charge $-4e/3$ would be a good candidate[8,18] for catalysing proton and deuteron fusion. If there is a general trend for $(3A+2)$ quarks to be more stable than $(3A+1)$ quarks, one expects systems such as u^5d^6 (charge $+4e/3$) to be more frequent than quarklei[17] such as u^6d^7. In such a case, both the adducts $q^2(\bar{q})^3$ and $q^3(\bar{q})^2$ (where q is a quark) are stable against internal annihilation forming single quarks. Another way of regarding these two systems is to let the primordial anti-quark or quark polarize vacuum to add two mesons.

It is not evident that a defective α-particle u^5d^6 is lighter than a defective ^{40}Ca nucleus $u^{59}d^{60}$. Hence, spontaneous growth may occur along the lines of

$$u^5d^6+[u^{54}d^{54}+(\bar{u})^{54}(\bar{d})^{54}]\rightarrow u^{59}d^{60}+(\bar{u})^{54}(\bar{d})^{54}\qquad(2)$$

the anti-argon nucleus ^{36}Ar gaining sufficient kinetic energy to escape before annihilation with positive nuclei. Like long-lived technicolour hadrons[19] (with atomic weights between 10^2 and 10^6) X^- may build a low concentration[8,20] of the apparent actinium isotope ^{232}ThX shortly after the big bang (because the apparent lithium isotope ^{8}BeX is not radioactive like ^{8}Be and hence provides no bottleneck hampering nucleosynthesis[21]) and the reactions of type (2) may proceed rapidly. At once, a single anti-quark may also produce a heavy nucleus lacking one quark. The higher limit of Z is likely to be due to short-lived spontaneous fission. The lack of one quark should prolong the half life strongly (due to decreased fission energy) and shift the obtainable Z-values in the direction of 164. Nevertheless, there will be a border somewhere, and it is conceivable that the fixed carrier of fractional charge in niobium does not have $Z=40.666\ldots$ (such as an adduct with one d-quark) but rather Z-values close to 73 (tantalum), 91 (protactinium) or 105, for

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that matter. The scarcity and highly positive charges of such systems prevent their fusion to conventional nuclei.

For many reasons, it is important to look in minerals for species with anomalous high atomic weights. Some may have the Z-values known from chemistry[8,20] (also if long-lived exotic quarks with integral charges are feasible) and others, with effective charges $(Z+\frac{1}{3})$ or $(Z+\frac{2}{3})$, cannot be fully neutralized by electrons and are expected to show extensive geochemical fractionation[7,17,22]. The calutron plates of Oak Ridge (where at least 10^{30} nucleons have passed through a mass spectrograph in the form of 1,600-kg uranium containing 11-kg ^{235}U) may be the best available source[8]. It was recently suggested[23] that concentrations of u^7d^6 of $\sim 10^{-15}$ in the stellar interiors may solve the solar neutrino problem, a cyclic process involving u^9d^7 (not losing a nucleon like ^5Li and ^5He) replacing the Bethe CNO cycle around 14×10^6 K.

As far as the full interaction between the quarks in a nucleus goes, it does not seem possible[24] to use additive two-particle potentials (satisfactory in individual hadrons) and the dramatic variation of rest masses between systems having zero, one or two unsaturated quarks is more similar to residual masses of gravitational singularities[1], the overall attraction almost entirely cancelling the huge rest masses of the isolated quarks. If such a scenario is relevant, we have lost all chemical connotations of 'constituent' with the idea of roughly additive ground state masses.

Received 11 March; accepted 2 September 1983.

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Phase intergrowths in spinelloids

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The abundance of the olivine and spinel structures in the Earth's mantle has led to extensive research into the nature of their stability. An intermediate phase (β) in the transformation of olivine (α) to spinel (γ) has been observed for several M_2TO_4 systems[1–4]. Studies of the Ni_2SiO_4–$NiAl_2O_4$ system have found several additional phases[5], named spinelloids. The structure of each phase has been determined and found to be closely related to the spinel crystal structure. We report here preliminary results of a high resolution transmission electron microscopy (HRTEM) study of phase intergrowths in the nickel aluminosilicate spinelloids. We observe substantial phase intergrowth in all spinelloids thus far studied.

In both olivine and spinel structures tetrahedral TO_4 groups are isolated. Intermediate phases so far observed show a transition from olivine to low-pressure spinelloids (phase 1 and phase 2) having triple groups of corner-shared tetrahedra (T_3O_{10}). Phase 1 crystallizes in aluminate-rich samples. As pressure increases these phases transform to structures containing two corner-shared tetrahedra (T_2O_7) and isolated tetrahedra. Thus in phase 3 (β phase) only T_2O_7 groups are found and in the higher pressure phases, 4 and 5, tetrahedra become isolated such that T_2O_7 and TO_4 groups are systematically intergrown[5]. At pressures in excess of 90 GPa phase 5 transforms to spinel ($+NiO+Al_2O_3$) with its characteristic 'isolated tetrahedra' structure.

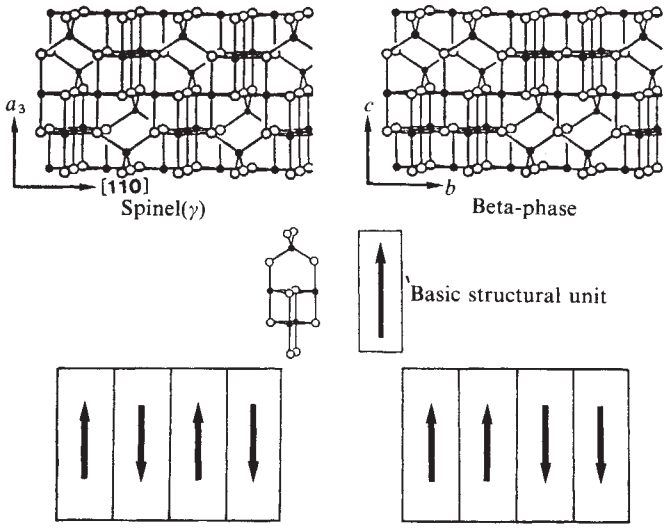

Fig. 1 Definition of the basic structural unit of the spinelloid structure. Perspective views of spinel and β-spinel (phase 3) structures are shown together with the arrangements of the basic structural unit in both structures. (Taken from Horiuchi *et al.*[5]).

Crystallographic relations between phases have been described by two different notations. Hyde *et al.*[6] use a description involving periodic unit cell twinning of the spinel structure to generate all the intermediate phases. They conclude the spinelloids may be considered as structurally intermediate between spinel and a hypothetical 'ω' structure. The ω structure has the highest possible density of twins. Horiuchi *et al.*[5] use a slightly different notation. They define a basic structural unit of an isolated tetrahedron and octahedron (see Fig. 1) and generate the spinelloids by different one-dimensional arrangements of these units (see Table 1).

This paper summarizes HRTEM studies of phase 2, phase 3 (β-phase) and phase 4. All samples were studied using a JEOL 200 CX electron microscope. Synthesis conditions are summarized in Table 1, the high pressure samples were prepared using a piston-cylinder and tetrahedral anvil apparatus. Powdered samples were used for microscopy by dispersal on a holey carbon grid. Characterization by powder X-ray diffraction showed samples to be largely one phase although traces of olivine were found in phase 2 and 3 and a trace of phase 3 in phase 4.

To differentiate between projected octahedra and tetrahedra, lattice imaging was conducted down the [100] zone axis of the orthorhombic spinelloids. Other orientations were investigated and individual results for each phase will be described elsewhere (P.K.D. and M.A., in preparation).

For all phases considerable microstructural intergrowth was observed. Diffraction patterns indicated substantial disorder in the b direction of the lattice, and few grains showed no multiphase assemblage.

Figure 2 shows images for phase 4; corresponding diffraction patterns are inset. For image interpretation calculated images for several defocus values and crystal thicknesses were compared with crystal potential projections. The calculated image for

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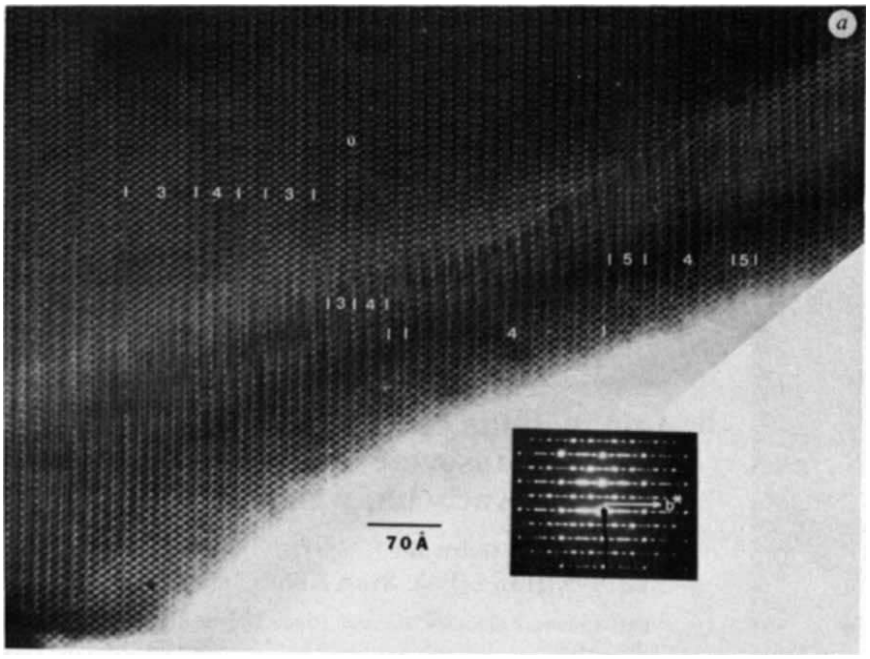

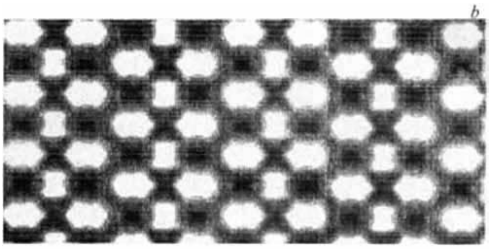

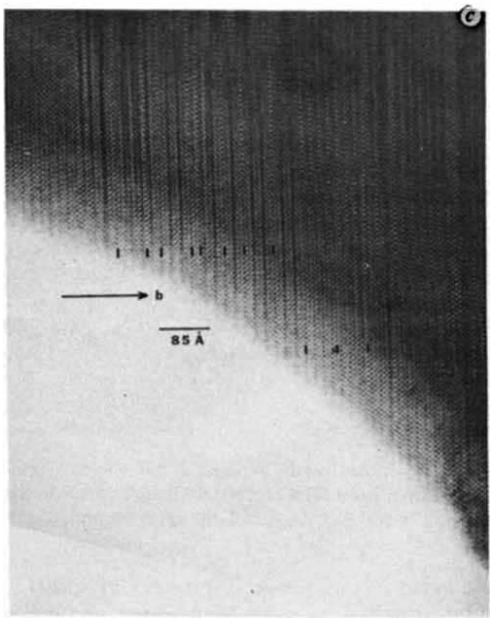

Fig. 2 Calculated and experimental structure images of phase 4. *a*, Image down the [100] zone axis showing intergrowths of phases 3, 4 and 5. The corresponding diffraction pattern (inset) shows considerable disorder in the b* direction. *b*, Calculated image for phase 4 at a defocus of 800 Å. Large black regions correspond to two adjacent octahedra, smaller dark regions to single octahedra. *c*, In this grain the left part of the image shows an intergrowth of (21) and (2) units, giving rise to longer repeats than are observed in the right-hand part of the image which is largely phase 4.

phase 4 is shown in Fig. 2*b*. Using this orientation lattice images can clearly distinguish between octahedral and tetrahedral sites. Using the notation of Table 1, phase 4 has a 221221-type structure. In the image adjacent octahedra appear as dark spots, single octahedra as slightly greyer 'dots'. Analysis of the alternation of these two structural units allows interpretation of microphase regions in each grain. Figure 2*a* shows regions of phase 4-type structure plus several intergrowths of phase 3-type structure (2,2-β phase). In addition intermediate slabs of phase 5-type structure (21) may be discerned. Intergrowths of these other structure types are supported by diffraction information. Discrete superlattice reflections suggest structure-types of 10 periodic units (phase 4), 4 periodic units (phase 3) and either a 4-periodic unit with a primitive cell (phase 1-31) or an 8-periodic unit with a body centred cell (211,211-a phase previously not observed). Quadrupling of the lattice vector in the (010) direction was a fairly common feature. The (211211) structure is the most likely since T_3O_{10} groups were not observed in any images of phase 4. Thus only intergrowths of T_2O_7 and TO_4 groups occur; no doubt this is a reflection of the unfavourability of three corner-sharing tetrahedra at such high pressure.

Figure 2*c* is of a different grain. The characteristic (221221) sequence of phase 4 is identifiable. However, the mid-part of the image reveals an intergrowth of the structural units (21) and (2). Phase 4 could be viewed as an ordered alternation of these units resulting in the (221221) structure. However, in this region of the grain more complicated sequences occur. What at first may appear as a random intergrowth of (21) and (2) units in fact shows four repeats of a sequence 22121212121.

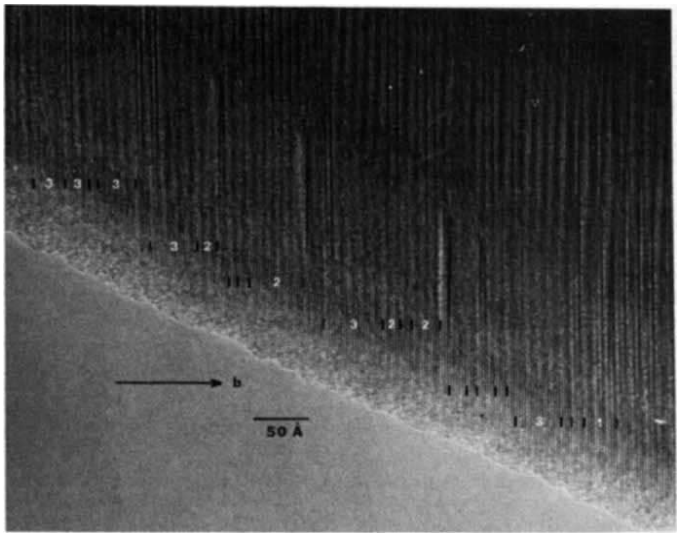

Fig. 3 Image of phase 3, β-spinel, showing intergrowths of phases 3(22), phase 2(33) and phase 1(31).

Table 1 Synthesis conditions for phase 2, 3, 4 materials

Phase	Composition	Stacking sequence	Pressure of synthesis (GPa)	Phases to X-rays	'Phases' in HRTEM
2	$3NiAl_2O_4:2Ni_2SiO_4$	33	0.0001	2+ trace olivine	1(31); 2(33); 3(22); 4(221221)
3	$NiAl_2O_4:Ni_2SiO_4$	22	2.6	3+ trace olivine	1(31); 2(33); 3(22); 4(221221); (32)
4	$NiAl_2O_4:Ni_2SiO_4$	221,221	6.0	4+ trace 3	3(22); 4(221221); 5(21); (211211); intergrowth of (21) and (2)

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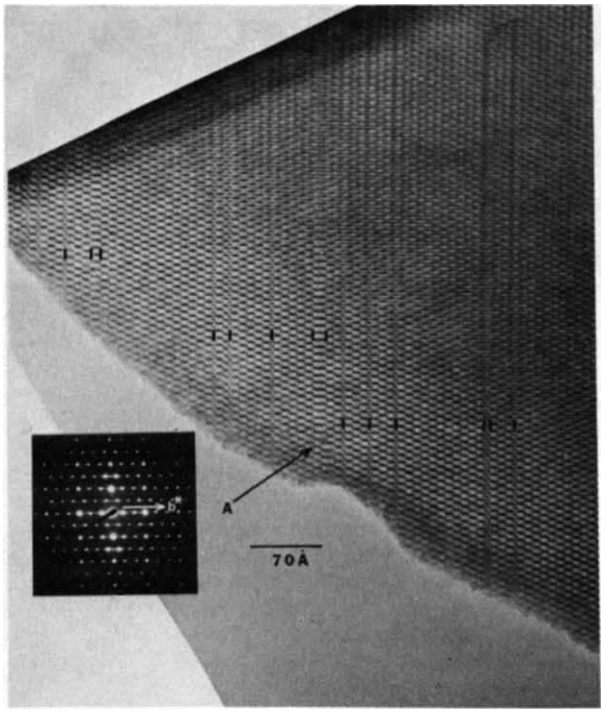

Fig. 4 Intergrowths in phase 2(33); the corresponding diffraction pattern is inset. The region labelled A corresponds to the sequence 63 changing to 36.

Figure 3 is an image of phase 3, β-spinel (22). Again many intergrowths are visible. Microstructure types identifiable in this region of the crystals include phase 3 (22), phase 1(31) phase 2 (33), with intermediate slabs of (32) and (21) commonly observed. Diffraction patterns show discrete reflections corresponding to phases 4, 3 and 2, plus considerable disorder. Thus this 'intermediate pressure' phase shows disorder involving sequences of T_3O_{10}, T_2O_7 and TO_4 groups.

The low-pressure phase, phase II is shown in Fig. 4. Again many intergrowths are observed. Interesting faults are produced by alternation of intergrowths; thus in the region labelled A the sequence (63) changes to (36). This results in a region of overlap with chains of edge shared octahedra sharing corners, that is, a portion of the rocksalt structure. Structure-types identified from lattice images include phase 2 (33), phase 1 (31) and phase 4 (221221). Diffraction patterns confirm observations of phases 2 and 1 and also show reflections from the β-phase. Again most patterns show considerable streaking along b^*.

In conclusion all spinelloid phases investigated show substantial disorder. The intergrown structure types are mainly those of a higher or lower pressure spinelloid phase. The extent of phase disorder appears independent of the synthesis conditions, thus phase disorder resulting from pressure quenching does not seem to be the cause of the intergrowths. New stacking sequences on a microscopic level have been observed. The occurrence of some of these is greater than that expected for a purely random arrangement of structural units and they could be defined as unique structure types. Such intergrowths are not surprising considering the structural similarity of the spinelloid phases. Calorimetric studies of spinelloid 'X-ray phases' (M.A. and A. Navrotsky, in preparation) show energetic differences are small, thus no one phase is greatly stabilized relative to another. Perhaps the surprising aspect of this work is the stability of the structural arrangements of the 'X-ray' phases. Thus certain structural sequences (for example (221221), (21), (31)) are much more common than a random sequence or other ordered sequences (such as (32), (22121)). Presumably the reasons are primarily energetic and coulombic-type structure modelling would seem worthwhile. This work also raises doubts as to the one-phase nature of the β-spinels in other systems (for example, Mg_2SiO_4, Co_2SiO_4). High-resolution studies of these systems will be undertaken with a view to characterizing any microstructural changes that may occur.

We thank J. Susaki (University of Tokyo) for synthesis of phase 4 samples, and R. M. Hazen for a constructive review. The electron microscopy was performed at the High Resolution Electron Microscopy Facility at Arizona State University.

Received 14 June; accepted 26 August 1983.

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Implications of new data on Mesozoic rocks of Kachchh, western India

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A lack of detailed facies studies has resulted in a false impression of the Mesozoic depositional setting in Kachchh, western India (previously, Kachchh has been termed 'Kutch' or 'Cutch'). In addition, designation of part of this sequence as Gondawana implies, by tradition, a predominantly non-marine origin. Recently, we examined these Jurassic–Cretaceous units and found numerous wave-built sedimentary structures that have been previously overlooked or unreported, abundant marine trace fossils, and highly bioturbated and glauconite-rich beds. Thus, we now propose a marine origin for the entire Kachchh succession. This interpretation has important implications regarding similar sequences around the Indian plate margin.

Jurassic–Cretaceous sedimentary rocks of Kachchh (Table 1) unconformably overlie a Precambrian basement and crop out in east–west trending anticlines (Fig. 1). Early investigators[1–4] provided a twofold stratigraphic classification: marine fossil-bearing rocks as 'Lower Jurassic Group' and the overlying plant-bearing beds as 'Upper Jurassic Group'. Waagen[5,6] adopted a fourfold classification of Patcham, Chari, Katrol and Umia 'Groups' in ascending order. Discovery of Aptian ammonoids[6] extended the age of the succession into Lower Cretaceous. The first three Groups and the marine fossil-bearing 'lower part of Umia' corresponded to the Lower Jurassic Group and the plant-bearing 'upper part of Umia' constituted the Upper Jurassic Group of Wynne[4]. Subsequent workers[7,8] included the Aptian rocks in the Umia Group and assigned Wealden Age to the plant-bearing upper part of the Umia Group. Raj Nath[9] considered all plant beds to be post-Aptian and grouped them separately (Bhuj Series/Stage). In recent years a new rock-stratigraphic classification[10,11] has been made and Bajocian and Bathonian ammonoids[12] have been found in the Patcham Formation.

Depositional facies assigned to these units have varied. The Chari, Katrol and lower Umia Formations have been considered as shallow shelf whereas the plant-bearing upper Umia Formation has been referred to as non-marine 'Gondwana' and 'Coastal Gondawa'[13] or locally intertidal[14]. The Bhuj Member has been considered intertidal[14] and also continental[15]. More recently, the upper Umia Formation has been described as fluvial in eastern and deltaic in western Kachchh[11].

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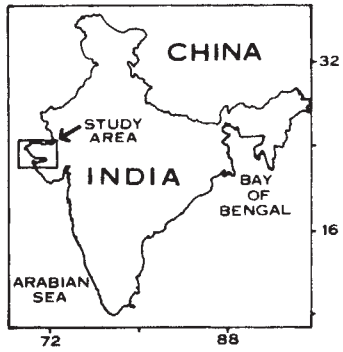
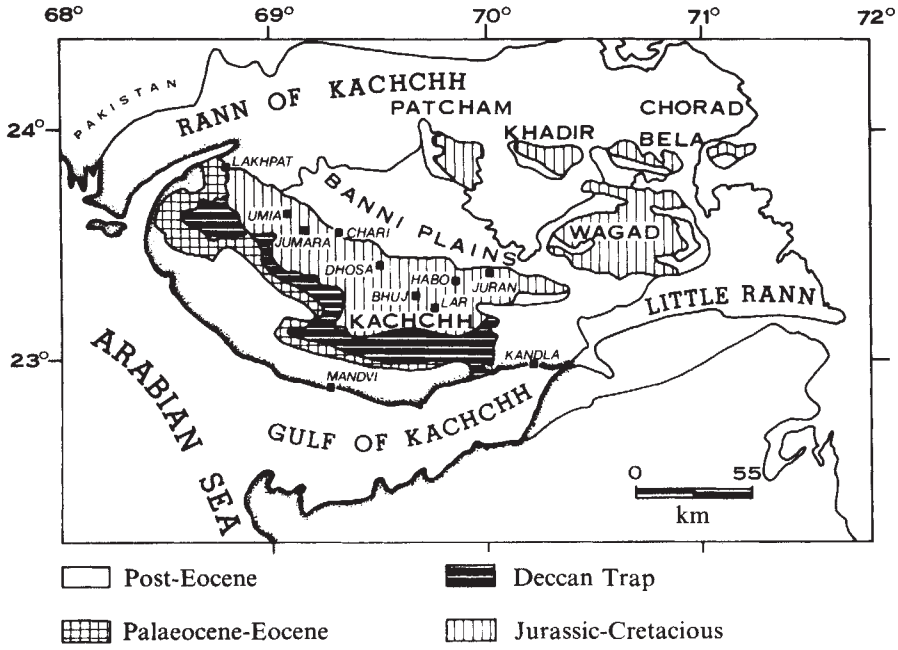


Fig. 1 Location of Kachchh study area (modified after Wynne[4]).



We examined nine sections of Mesozoic rocks in Kachchh for physical and biogenic structures and distribution of body fossils. These data establish a palaeoenvironmental framework in conjunction with stratigraphic control based on ammonoids and nannoplankton.

A general description of lithology, body and trace fossils is as follows: The Patcham Formation includes sand/shale alternations with bioclastic conglomeratic beds in the lower part and an upper part of limestone/shale alternations. Bivalves and brachiopods are the dominant macroinvertebrates. Vague horizontal trails and a few *Rhizocorallium* are the only trace fossils.

In the Chari Formation the principal sediments are thinly laminated clays with interbedded hard, ferruginous sandstone and sandy limestone bands, and a few intraformational conglomerates. This unit abounds in macroinvertebrates, dominated by bivalves, with diversified and well preserved ammonoids. Feeding, locomotion and dwelling traces are abundant and include *Thalassinoides*, *Ophiomorpha* and *Chondrites*, pholad borings and ammonoid resting marks.

Sediments of the Katrol Formation include marls and sandstones in the lower part, flaggy sandstones with subordinate shales in the middle part and coarse sandstone in the upper part. Body fossils are less abundant and less diverse than in the Chari Formation and are poorly preserved. There are many trace fossils, dominated by *Thalassinoides*, *Ophiomorpha* and *Rhizocorallium* with *Aulichnites*.

The Umia Formation in western Kachchh contains ammonoid-bearing glauconitic and oolitic sands, shales and sandstones containing trigonids, horizons with plant fragments, carbonaceous shales and coal, glauconitic shales and sandstones and thick sandy successions. Abundant fossil logs are present in the Ukra Member. In eastern Kachchh the Umia Formation is locally characterized by sandy successions rich in plant fossils and lacking marine body fossils. The entire Formation, both east and west, however, contains abundant marine trace fossils including *Ophiomorpha*, *Thalassinoides*, *Skolithos*, *Chondrites*, three types of *Rhizocorallium*, *Aulichnites* and *Cylindrichnus*.

Throughout the Mesozoic section in Kachchh, ammonoids are useful in dating and establishing inter-formation boundaries, and for providing clues to the cyclic fluctuations and geometry. The oldest ammonoids are Bajocian and Bathonian index forms from the lower part of Patcham Formation[12]. *Macrocephalites* dominates the lower Chari Formation and an endemic mayaitid assemblage (Upper Oxfordian) occurs in its youngest part, and suggests Upper Bathonian to Upper Oxfordian time span. The Katrol Formation is considered Kimmeridgian (?) or Lower Tithonian to at least middle Tithonian on the basis of virgatosphinctin fauna (*Torquatisphinctes*, *Pachysphinctes*, *Subdichotomoceras* and *Katroliceras*) in the lower part and extremely scarce *Hildoglochiceras* in the middle part; the upper Katrol is devoid of ammonoids. The lower Umia Formation in western Kachchh (Umia Member) contains *Virgatosphinctes* (mostly *Virgatosphinctes densiplicatus*) and *Micracanthoceras*

Table 1 Jurassic–Cretaceous stratigraphic succession in Kachchh

Lithostratigraphic units		Approximate thickness (m)	Lithology	Revised ages
Umia Formation	Bhuj Member Ukra Member Ghuneri Member	600	Thick, medium to coarse, often current-bedded sandstone, with thin shale and interspersed glauconitic and oolitic bands	Upper Tithonian–Albian
Katrol Formation		450	Sandstone and shales	Kimmeridgian?–Lower to middle Tithonian
Chari Formation	(Dhosa Oolite Member at top)	240	Shales with interspersed hard calcareous sandstone; limestone bands with concretionary nodules	Upper Bathionan–Upper Oxfordian
Patcham Formation		360	Limestone with subordinate shales and sandstones	Bajocian–Bathonian

The Umia Formation is differentiated in members in north-west Kachchh only and elsewhere in the mainland it is undifferentiated above Katrol Formation. Duplication of the term Umia for two ranks of lithostratigraphy (Formation and Member) stems from the original work of Raj Nath, and needs replacement at Member level. The Dhosa Oolite member occurs at the top of the Chari Formation all over Kachchh mainland. Patcham Formation is present only in Patcham 'Island' (north central area of Fig. 1) and Jumara Dome.

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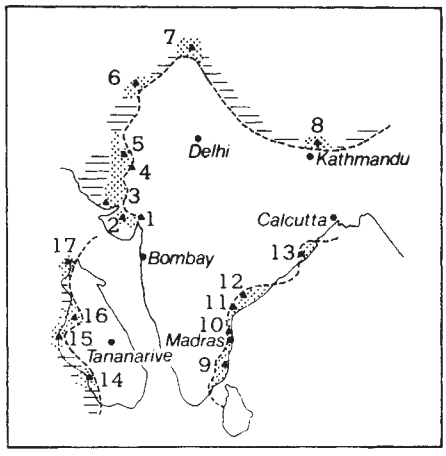

Fig. 2 Jurassic–Cretaceous sedimentary units around the Indian plate-margin (inclusive of Madagascar) with locations of so-called Coastal Gondwanas (intercalated with marine sediments, ▩). ▤, Undisputed marine sediments. Locations are: (1) Ahmadnagar Sandstone of Ahmadnagar; (2) Than plant beds of Than; (3) Bhuj Formation of Kachchh; (4) Barmer Sandstone of Barmer; (5) Parihar Formation of Jaalmer; (6) Lumshiwal Formation of Salt Range (Pakistan); (7) plant beds of Ladakh; (8) Kagbeni Sandstone of Thakkola (Nepal); (9) Sivaganga Formation at Ramnud (Cauvery Basin); (10) Sriperumbudur Formation bands and Sattavedu Formation of Madras (Palar Basin); (11) Gollapille Formation and Tirupati Formation between Rajahmundhri and Ellore (Krishna Basin; (12) Badavada Formation and Pavlur Formation between Ongole and Guntur (Godavari Basin); (13) Athgarh Formation of Orissa; (14) Morondova; (15, 16) Majunga; and (17) Diego.

of early late Tithonian Age. The Ukra Member (Umia Formation) is dominated by Aptian *Australiceras*. Furthermore, our new finds of Lower Albian *Cleoniceras* and *Lemuroceras* extend the Ukra into Lower Albian.

Abundant coccoliths occur in the Patcham Formation but recrystallization has destroyed their identity. The Chari Formation reveals excellent to moderately good preservation of coccoliths, whereas the Katrol and Umia Formations were barren of nannofossils. Nannoflora of the lowermost Chari Formation has proven to be the best known in the world. Several European middle Jurassic marker species are common in Kachchh and most exhibit extended vertical ranges. All species of *Stephanolithion*, with the notable absence of *Stephanolithion bigoti*, are present and thus suggest a late Bathonian age for the basal Chari Formation. The genus *Corollithion* and related genera make a sudden and abundant appearance at a level coinciding with reduced frequency of *Stephanolithion hexum* and rare *Stephanolithion octum*. Other useful species belong to *Striatococcus, Tubirhabdus, Podorhabdus, Polypodorhabdus, Diazmatolithus, Cyclagelosphaera, Schizosphaerella, Discorhabdus, Ethmorhabdus, Zeugrhabdotus* and *Watznaueria*.

Repetition of lithological units and fossil beds suggests repeated shallowing-upward cycles of sedimentation related to sea-level fluctuations. Transgressive phases are mostly non-depositional or erosional whereas regressive phases are depositional. A gradual increase of sand from the base of Katrol Formation, probably related to increased supply of terrigenous clastics, suggests faster sedimentation and subsidence. In the upper Katrol Formation, grain size and bed thickness increase. Sandy successions of the Umia Formation are commonly capped by a densely burrowed and bioturbated coarse sand, indicating periods of reduced terrigenous clastic supply.

Throughout the Mesozoic section of Kachchh we found evidence of deposition on a shallow, tide-affected shelf and in adjacent tidal flats. Sedimentation took place above the wave base, with tidal influences indicated by abundant wave-built structures including hummocky bedding, wave ripples and interference ripples. Estuarine channels and lagoons played a significant part as sites of deposition during early Cretaceous time.

The Patcham Formation represents deposits of a shallow transgressive sea with numerous fluctuations. The lower and middle Chari Formation contains abundant marine invertebrates and records the maximum depth of all the units studied (inner neritic shelf). The lower Chari Formation indicates high energy, as reflected by development of oolites. Beginning with the upper part of Chari Formation, the overall vertical succession largely consists of regressive shallow marine sequences (offshore mud and coastal sand interbedded with tidal flat deposits) indicating gradual emergence. Coal and carbonaceous shales in the middle Umia Formation represent coastal lagoons.

Clastic sequences similar to those in Kachchh occur around the Indian plate margin as well as elsewhere in Gondwanaland (Fig. 2). These are successions of similar age (mostly late Jurassic to early Cretaceous) and stratigraphic setting and as in Kachchh, have traditionally been considered non-marine or referred to as Coastal Gondwana. Based on our investigation and the regional similarities described above, we urge that the so-called non-marine coastal Upper Gondwana sediments be reexamined in the light of modern facies interpretation and concepts of sedimentation. Use of the term Gondwana, with its implication of inland fluvial sedimentation, should be abandoned for such coastal marine deposits. We stress that the presence of plant fossils and the absence of readily recognizable marine body fossils do not necessarily indicate non-marine sedimentation; in sandy clastic sediments of coastal areas, particularly of warm climate and protected sub-environment, shells are quickly leached out. Primary facies characteristics such as physical and biogenic sedimentary structures must invariably be considered.

We acknowledge the assistance of the District Magistrate of Kachchh, the Gujarat Department of Geology and Mines and the Geological Survey of India. Funding for travel to and within India (of J.D.H.) was provided by the NSF, The American Institute of Indian Studies, University Grants Commission and the Committee for Research and Exploration of the National Geographic Society.

Received 8 August; accepted 25 August 1983.

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$^3He/^4He$ ratios in CH_4-rich natural gases suggest magmatic origin

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Natural gas and oil fields, commercially exploited since the 1950s, are distributed in the zonal area facing the Sea of Japan in north-east Japan. We report here measurements of the $^3He/^4He$ ratios in 27 CH_4-rich natural gases obtained from commercial oil and gas wells in the area. We observed extraordinarily high $^3He/^4He$ ratios, up to 8.65×10^{-6}, in gases from wells drilled into deep reservoirs in volcaniclastic rock formations, the so-called 'Green Tuff' (Fig. 1). These values are almost identical to those of volcanic fumaroles in the Japanese Islands. In contrast, gases in shallower sedimentary rock reser-

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voirs have low $^3He/^4He$ ratios with a minimum value of 0.3×10^{-6}. These low values are similar to those of natural gases originating from biological materials. This suggests that the formation of natural gas reservoirs in the volcaniclastic rock may be attributed to large-scale magmatic activities which occurred in the middle Miocene.

The characteristic greenish colour of the altered volcanic rocks in the Green Tuff is considered to be a result of diagenetic and hydrothermal alterations. Volcanic and sedimentary piles up to several thousand metres thick distributed in the region are attributed to subsidence and subsequent submarine volcanism in the middle Miocene[1].

It has been generally accepted that the CH_4 in natural gas is produced by the anaerobic bacterial decomposition of organic matter in an anoxic environment[2], so an inorganic origin for the CH_4 gas source has been regarded as hardly plausible. Hydrocarbon accumulation in volcanic rock reservoirs has generally been attributed to migration from sedimentary source beds. Downward migration of fluids may occur as a result either of subsidence of rock mass caused by fault movements or of abnormal reservoir compaction[3,4].

Nevertheless, outgassing of CO_2 and H_2 of inorganic origin is known to be associated with volcanic activity. Discharging of CH_4 and H_2, as well as He and Rn, was reported in hydrothermal fluid at 21° N on the East Pacific Rise and the Galapagos Spreading Centre[5,6]. The presence of CH_4 in popping rocks from the Mid-Atlantic Ridge has been reported[7,8]. Significant amounts of hydrocarbon gases (up to 120 cm^3 per kg rock) were also observed in the Khibiny and Lovozero massifs (Kola Peninsula), the Kiya-Shaltyr and Sredniy Tatar intrusions in Siberia, and the Ilimaussaq massif in Greenland[9]. The methane concentration of the gases reaches as much as 90%. Thus, primitive (inorganic) CH_4 should not be neglected as a possible source of the natural gases.

The $^3He/^4He$ ratio in natural gas may provide the most useful information on the origin of the gas. Most ^3He is primordial and is still being outgassed from the Earth's mantle as a juvenile component[10]. Most ^4He is radiogenic, produced by α decays of natural radioactive elements such as U and Th. Therefore, higher $^3He/^4He$ ratios in materials imply their closer relationship to the mantle materials.

The $^3He/^4He$ ratios in natural gas have been thought to be low in general. Aldrich and Nier[11] reported extremely low $^3He/^4He$ ratios for some natural gases in the United States, of 5×10^{-8} to 5×10^{-7}. Similar low values were reported for natural gases of the continental area in the Soviet Union[12], and Sano *et al.*[13] reported low $^3He/^4He$ ratios in natural gases collected from the southern Kanto District, in the arc-trench gap region of the Japanese islands (1 to 3×10^{-7}). The lower $^3He/^4He$ ratios are interpreted to be due to lack of magma genesis in the region and to accumulation of radiogenic ^4He derived from decay of U and Th in the sediment during geological time. Low $^3He/^4He$ ratios for these natural gases would be expected where gases are of biogenic origin.

We collected 27 CH_4-rich fuel gas samples from commercial gas wells in the Green Tuff region. Samples were introduced into 50–ml lead–glass containers with vacuum stopcocks using a displacement method. Isotopic ratios of $^3He/^4He$ were obtained using a 6-inch magnetic deflection type mass spectrometer (6-60-SGA; Nuclide Co.). Ion beams of ^3He and ^4He were measured by a double-collector system. Resolving power of ~600 at 1% of peak height was attained for the complete separation of the ^3He beam from those of H_3 and HD. A precise description of the $^3He/^4He$ ratio measurement, including the purification procedure for He, has been published elsewhere[13]. The bulk chemistry of natural gas samples and their He concentrations were determined by gas chromatography[14].

Measured $^3He/^4He$ ratios varied widely between 3.41×10^{-7} and 8.65×10^{-6} (Table 1). The range overlaps with that of $^3He/^4He$ data on soil gases, spring gases and volcanic gases of Japan reported previously[13,15]. The lowest $^3He/^4He$ ratio, approximately one-quarter the atmospheric ratio (1.4×10^{-6}),

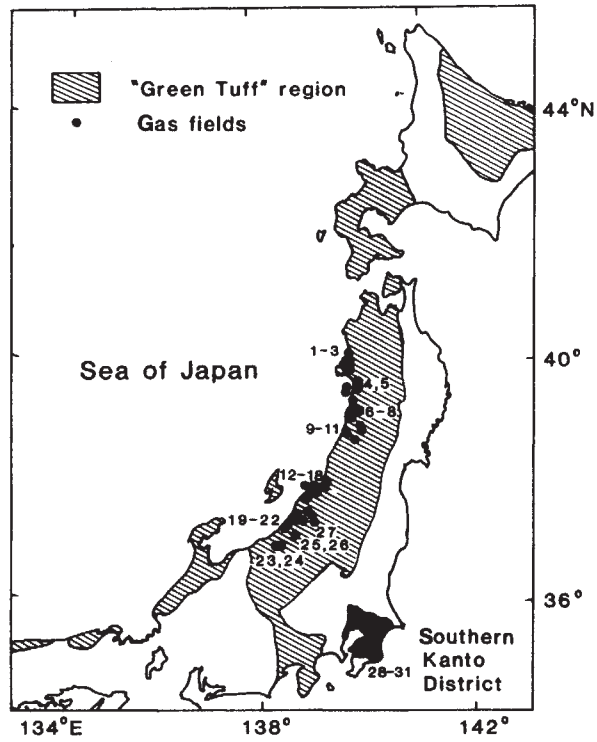

Fig. 1 A map showing the distribution of gas fields and the Green Tuff region. Approximate positions of sampling sites are shown (listed in Table 1).

is similar to that of He in CH_4-rich gas reservoirs in the southern Kanto District[13]. Gas reservoirs with low $^3He/^4He$ ratios in this study are mostly in sandstone. The highest value of 8.65×10^{-6}, six times the atmospheric ratio, is close to the values for volcanic fumaroles in the subduction zone which includes the Japanese Islands[15,16]. The highest ratio was observed for a gas reservoir in a volcaniclastic layer at 4,600 m depth.

Methane gases with higher $^3He/^4He$ ratios are found predominantly in altered volcaniclastic rock reservoirs but are also observed in some sedimentary layers, such as the Funakawa and Tentokuji Formations. No gases with low ratios are found in the volcaniclastic reservoirs such as the Nishikurosawa and Nanatani Formations. The major constituent of most gas samples was CH_4. Note that one sample at Fukura contains up to 68% CO_2; this might suggest its volcanic origin. Helium concentrations of CH_4 gas in volcaniclastic reservoirs are higher than those of sedimentary reservoirs.

The simplest explanation for such high $^3He/^4He$ ratios is that high permeability of He through such volcaniclastic materials resulted in preferential escape of He from a magmatic source to overlying biogenic natural gas reservoirs. Alternatively methane with such a high $^3He/^4He$ ratio might originate from magmatic gas. Reequilibration of CO_2 and H_2 of volcanic origin will form CH_4 in certain conditions of pressure and temperature. The reaction

$$CO_2+4H_2 \rightleftharpoons CH_4+2H_2O$$

proceeds to the right under increased pressure and decreased temperature. Karzhavin and Vendillo[9] explained that observed high CH_4 concentration in fluid inclusion in massifs can be formed in a magma in conditions of thermodynamic equilibrium. Halloway[17] showed increased concentration of CH_4 under 2 kbar and 750 °C, computed on the basis of real gas behaviours of volcanic gases.

Observed $^3He/^4He$ ratios in natural gases may be attributed to an admixture of two gases from different sources: purely biogenic CH_4 with low $^3He/^4He$ ratio, and CH_4 with high ratio formed by purely inorganic reactions of gases of volcanic origin as shown above. In such a case, not more than 30% of CH_4 of volcanic origin can be mixed with the biogenic CH_4 (see below).

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Table 1 $^3He/^4He$ ratios of CH_4-rich natural gases in Japan

No. Location	Depth (m)	Gas reservoir Formation	Rock type	$^3He/^4He$ (10^{-6})	CH_4† (%)	CO_2† (%)	N_2† (%)	He† (p.p.m.)
Green Tuff region								
1. Yoshino, Akita	948	Tentokuji	Sedimentary	4.12±0.03	99	0.37	0.68	11
2. Fukumezawa, Akita	1,330	Onnagawa	Sed./ign.	6.24±0.07	68.3	1.31	0.37	4
3. Fukumezawa, Akita	1,280	Onnagawa	Sed./ign.	5.96±0.12	73.2	1.04	0.46	4
4. Yabase, Akita	1,300	Onnagawa	Sed./ign.	1.18±0.04	66.1	0.006	0.24	2
5. Omonogawa, Akita		Onnagawa	Sed./ign.	1.42±0.08	77	1.22	0.23	2
6. K-Yurihara, Akita	1,429	Nishikurosawa	Igneous	7.25±0.13				
				7.13±0.08				
7. Yurihara, Akita	685	Tentokuji	Sedimentary	2.45±0.05				
				2.54±0.05				
				2.39±0.04				
8. Kisagata, Akita	300	Tentokuji	Sedimentary	2.42±0.03	97.3	4	2.4	4
9. Fukura, Yamagata	823	Kitamata	Ign./sed.	6.87±0.17	29.5	67.5	0.67	30
10. Amarume, Yamagata	1,010	Kitamata	Ign./sed.	6.39±0.13	82.6	1.7	0.29	2
11. H-Amarume, Yamagata	616	Maruyama	Sedimentary	7.77±0.15	99.3	0.94	1.5	47
12. Aga-oki, Niigata	2,167	Shiiya	Sedimentary	1.90±0.02	93.4	0.89	0.14	2
13. Aga-oki, Niigata	2,052	Nishiyama	Sedimentary	0.746±0.042	99	0.70	0.15	2
14. Aga-oki, Niigata	2,170	Nishiyama	Sedimentary	0.341±0.020	89.6	0.83	0.13	2
15. M-Aga, Niigata		Shiiya	Sedimentary	3.76±0.08	82.1	0.49	1.0	4
16. M-Aga, Niigata		Shiiya	Sedimentary	3.08±0.02	76.8	0.27	4.9	2
17. Matsuzaki, Niigata	3,000	Shiiya	Sedimentary	3.22±0.09	94.6	0.89	0.2	3
18. Matsuzaki, Niigata	3,000	Shiiya	Sedimentary	3.23±0.03	88.8	1.1	0.1	4
19. H-Kashiwazaki, Niigata	2,600	Nanatani	Igneous	7.69±0.08	94.2	0.068	2.3	58
20. H-Kashiwazaki, Niigata	2,600	Nanatani	Igneous	6.98±0.10	76.8	0.50	0.72	
21. H-Kashiwazaki, Niigata		Nanatani	Igneous	7.33±0.09	93.8	0.14	2.4	51
22. H-Kashiwazaki, Niigata		Nanatani	Igneous	6.90±0.10	60.7	0.19	0.5	
23. Meiji, Niigata	1,350	Teradomari	Sedimentary	3.10±0.06	98	0.91	0.59	5
24. Bessho, Niigata	1,620	Teradomari	Sedimentary	2.09±0.08	98.2	0.18	0.61	6
25. K-Katagai, Niigata	4,646	Nanatani	Igneous	8.65±0.08	80.9	6.1	2.8	31
26. Katagai, Niigata	1,022	Nishiyama	Sedimentary	5.59±0.08	91.3	0.56	0.54	10
27. Mitsuke, Niigata	1,821	Nanatani	Igneous	8.61±0.11				
Southern Kanto District								
28. Narashino, Chiba	1,700	Kazusa	Sedimentary	0.213±0.006*	99.6	1.47	0.14	<0.5
29. Yokoshiba, Chiba	1,224	Kazusa	Sedimentary	0.349±0.011*	89.6	3.23	0.66	<0.5
30. Shirako, Chiba	493	Kazusa	Sedimentary	0.310±0.005*	98.7	0.72	0.32	1
31. Chonan, Chiba		Kazusa	Sedimentary	0.221±0.010*	97.8	0.26	2.2	0.7
32. Heiwajima, Tokyo	900	Kazusa	Sedimentary	0.137±0.010*	95.6	1.17	1.12	3

Sed., Sedimentary; ign., igneous.

* Ref. 13. † Analysed by Urabe[14].

If we make the reasonable assumption that the $^3He/^4He$ ratios of biogenic CH_4 gas and magmatic gas are 10^{-7} and 10^{-5}, respectively, and that magmatic gas contains 10 times as much ^4He as biogenic methane, the calculated mixing ratio should be 1:0.66 at the observed $^3He/^4He$ ratio of 8.7×10^{-6} in volcaniclastic rock reservoirs. If the above assumptions are valid, at least one-third of the natural gas in such volcaniclastic reservoirs is of magmatic origin. Mid-high $^3He/^4He$ ratio values may also be due to admixture of biological and magmatic CH_4 in different mixing ratios.

We therefore suggest that appreciable amounts of CH_4-rich gases in volcaniclastic rock reservoirs were derived from the upper mantle with He via magmatic activity. As only scattered volcanic activity occurred in the area in the Pliocene and Quaternary, a probable candidate is the volcanic activity associated with the Green Tuff magmatism in the middle Miocene.

According to neotectonic studies on the north-east Japan Arc, the stress field was tensional during the period from early to middle Miocene[18,19]. The tensional stress field in the region resulted in the development of large-scale grabens with a north–south direction, parallel to the present volcanic front. The tensional environment opened fractures and created high permeability in the area. Volcaniclastics erupted and were deposited throughout depressions mostly in submarine environments. By the process of diagenetic and strong hydrothermal alterations over geological time, gases originally contained in volcaniclastics would be released. The presence of both highly porous media of volcaniclastics and impermeable cap rocks might be effective in the formation of natural gas fields.

We thank Professor Kazuaki Nakamura of the University of Tokyo and Dr Takeo Sato of the Geological Survey of Japan for reading the original manuscript; Dr Motoaki Sato of the United States Geological Survey for critical comments; Drs Y. Nakamura and K. Notsu for useful discussion; Mrs A. Urabe for analysing the chemical composition of gases; Teikoku Oil Co. and Japan Petroleum Exploration Co. for gas sampling; and Mr T. Hyodo of JAPEX and Mr R. Hiwada of the Japan Natural Gas Association for arranging the sampling.

Received 17 June; accepted 26 August 1983.

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Discovery of an anoxic basin within the Strabo Trench, eastern Mediterranean

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We report here the discovery of a basin, which we call the Tyro Basin, in the eastern Mediterranean in which anoxic conditions exist and organic-rich sediments are accumulating. Bottom water samples show extremely high salt concentrations. A horizontal midwater sound reflector about 70 m above the basin floor was observed in a seismic reflection profile across the basin. From shipboard observations we conclude that dissolution of Messinian evaporites, exposed by faulting in the sides of the basin, causes stagnant bottom conditions. This basin provides an opportunity to test some of the hypotheses concerning the formation of sapropels present in cores collected from the same area. Cores taken in an adjacent similar basin (for which we propose the name Kretheus Basin) indicate a recent change from anoxic to oxygenated conditions which was probably caused by tectonic activity associated with the Strabo Trench.

The mode of accumulation of organic-rich sediments within the deep marine environment is of great interest in determining the controls of hydrocarbon source rock formation. Modern examples of the formation of these sediments have been encountered in the Black Sea, the California Borderland basins, the Orca Basin in the Gulf of Mexico and on the upper continental slope in the north-west Indian Ocean. These occurrences are all associated with anoxic conditions at the sediment–water interface. Such conditions were not known to exist within the eastern Mediterranean, although coring and deep-sea drilling in the region reveals at least 12 sapropels with organic contents of up to 16% within the Quaternary sedimentary section[1].

Table 1 Location, depth and length of cores

No.	Loran position	C(m)	Depth (m)	Length
Tyro Basin				
B8	33°52.31′ N	26°0.1.64′ E	3,358	0.54
P9	33°52.51′ N	26°00.68′ E	3,390	0.32
B44	33°52.02′ N	26°01.95′ E	3,415	0.44
P46	33°52.02′ N	26°02.30′ E	3,473	3.68
Kretheus Basin				
P45	33°50.54′ N	25°56.73′ E	3,316	2.66
B47	33′50.27′ N	25°57.13′ E	3,098	0.34
Ridge				
P48	33°50.10′ N	25°59.05′ E	2,724	1.86

P, Piston-core (diam. 9 cm); B, box-core (base 30×20 cm).

The presently anoxic Tyro Basin is one of several small basins with depths in excess of 3,300 m situated within the western end of the Strabo Trench (Figs 1, 2). Detailed topographic maps of the area are available as a result of multi-narrow beam echosounder 'Sea Beam' surveys carried out on the RV *Jean Charcot*[2]. The Strabo Trench forms the boundary between a seismically active transform section of the Hellenic Trenches and the relatively seismically inactive Eastern Mediterranean Ridge[3,4]. It is thought to be a younger feature than the Pliny Trench[5,6] to the north from which it is separated by an uplifted block containing thick evaporites[7]. Topographic trends within this part of the Strabo Trench are postulated to be the result of strike slip motion (see ref. 3, Fig. 8). The two basins investigated during our cruise are about 3 km wide and are separated by a sill at a depth of 3,100 m. Two box-cores and a 3.78-m long piston-core were recovered from the Tyro Basin. A box-core and 2.65-m long piston-core were taken in the Kretheus Basin, and an 1.86-m piston-core was obtained from a topographic high to the south (Fig. 2, Table 1). In addition to a detailed 3.5-kHz echosounding grid with a spacing of 2 km, several seismic reflection profiles were run. Navigation on the Tyro consisted of Loran C supported by satellite fixes.

The samples obtained from the Tyro Basin all indicate that anoxic conditions prevail in this basin. The bottom water and upper part of the sediment column smell strongly of H_2S. The uppermost sediment is a dark greyish-green gelatinous ooze, and the piston-core contains laminated greyish-green fine-

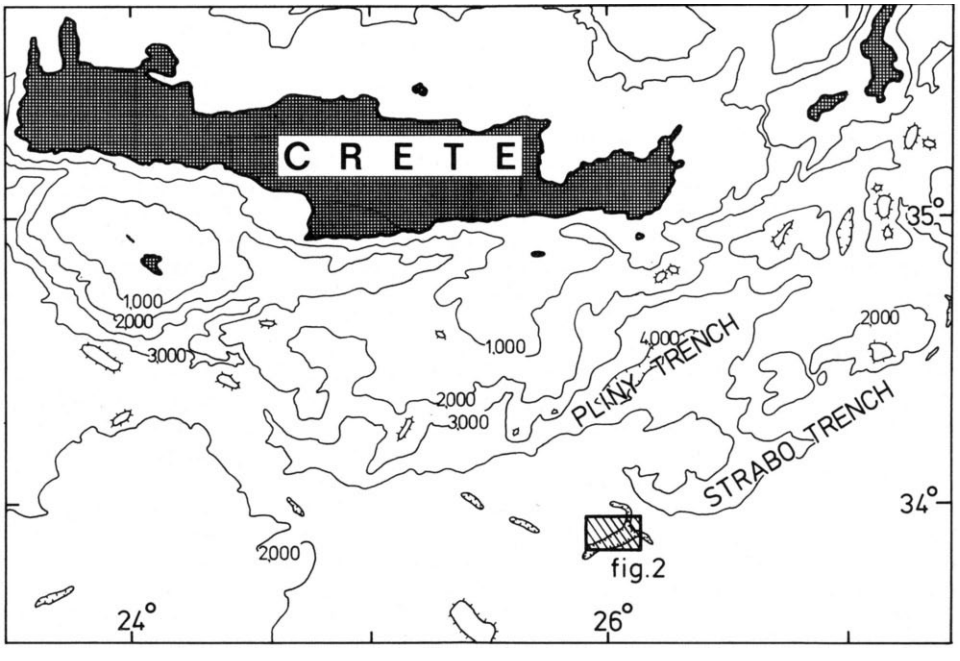

Fig. 1 Bathymetry of the region and location of the area shown in Fig. 2 in which an anoxic basin is present. (After IOC-International Bathymetric Chart of the Mediterranean[12].)

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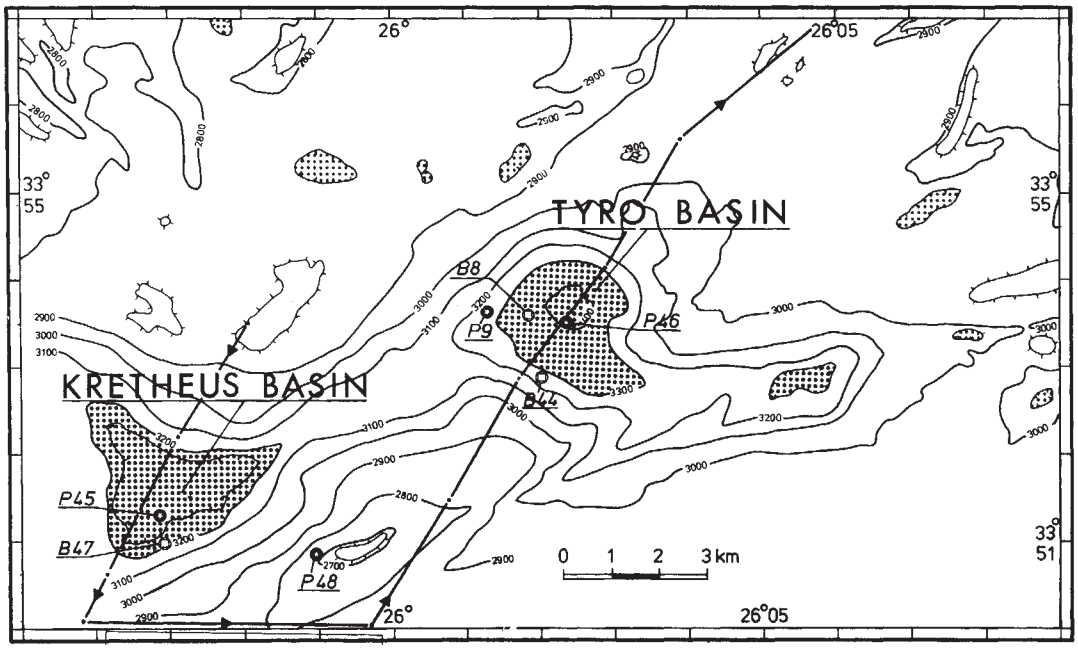

Fig. 2 Seafloor topography in the Western Strabo Trench based on Sea Beam surveying[2]. The Tyro Basin is anoxic at present, the Kretheus Basin is oxic. Locations of cores are preliminary and have been shown relative to the Sea Beam topography, which is based on satellite fixes. The locations based on Loran C navigation are shown in Table 1. The approximate position of the seismic reflection profile shown in Fig. 3 is indicated.

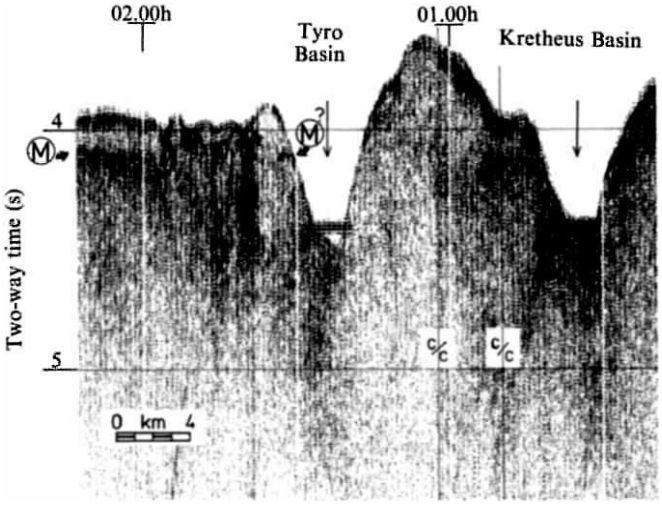

Fig. 3 Seismic reflection profile across the Tyro and Kretheus Basins. The profile shows a midwater horizontal sound reflector in the Tyro Basin at a depth of 4.37 s two-way time. The short reflector exposed in the side of the Tyro basin may be due to Messinian evaporites causing the 'M' reflector.

grained sediment with sapropelic layers and layers composed of pteropod and foram ooze. The samples taken from the Kretheus Basin show that it is presently oxygenated, as the uppermost 1–3 cm sediment consists of brown-coloured ooze. Below this ooze, the sediments in the pistoncore are the same greyish-green laminated oozes described for the Tyro Basin and may have been deposited in anoxic conditions. Piston-core P 48 (Fig. 2) on the ridge contains a characteristic eastern Mediterranean stratigraphy consisting of 32 cm of buff-coloured calcareous mud, overlying 9 cm of sapropel followed by greyish clay in which a tephra layer occurs at a depth of 70 cm. The sapropel layer may correlate with sapropel horizon S_1 dated at around 8,000 yr BP (ref. 1).

Geochemical investigations of the cores and the bottom water obtained in Tyro Basin[8] confirm the presence of anoxic conditions by oxygen depletion and extremely high salt concentrations in the bottom water. There is enrichment of ammonia in the pore water and of organic carbon in the sediment.

Preliminary faunal analysis of the uppermost 2 cm from the box-core sample B 8 in the Tyro Basin showed the presence of a rich and well preserved microfauna, composed predominantly of planktonic organisms. Radiolaria and diatoms are especially abundant. Planktonic foraminiferal species include *Globigerinoides ruber* (pink and white), *Orbulina universa*, *Hastigerina pelagica*, *Globigerina calida*, *Globigerina bulloides* and *Globorotalia scitula*. Juvenile specimens are common. Only a single benthonic form, *Fursenkoina* sp., was found. Pteropods include species of *Limacina*, *Cavolinea* and *Clio*. Small molluscs and otoliths are also present. A sample from the laminated bottom section of the same box-core at 50 cm reveals a different planktonic foraminiferal assemblage. *G. ruber* is still common but the pink types are rare. In addition to the species found in the top sediments, *Neogloboquadrina pachyderma* (dextral), *Globorotalia inflata*, *Globigerinoides sacculifer* and *Biorbulina bilobata* were encountered. Several specimens of the benthonic foraminiferal genera *Pyrgo* and *Triloculina* were also found. Interestingly, in this box-core there are thin bands of microlaminated dark-green rubbery sediment which seem to consist of strongly coagulated diatoms and algae.

The full implications of the discovery of this anoxic basin will emerge after the water samples, cores, acoustic and seismic data have been analysed. However, some preliminary conclusions can be made at this stage. First, anoxic conditions do occur locally in the eastern Mediterranean. Second, conditions possibly leading to the formation of sapropels may exist in the Tyro Basin. This contradicts current opinion that such conditions do not exist[9]. This specific environment in the Tyro Basin is related to high salt concentrations in the bottom waters, causing stratification in the water column, which is similar to processes observed in the Orca Basin in the Gulf of Mexico[10]. A seismic profile over the Tyro Basin (Fig. 3) shows that the 'M' reflector associated with the Messinian evaporites may be outcropping in the sides of the basin. The presence of salt diapirs has been postulated for the area of the Strabo Trench from Sea Beam[2] and side scan sonar (Gloria) surveying[6]. Further evidence for stratification of the water column in the Tyro Basin is provided by the presence of a horizontal midwater sound reflector at a depth of 3,350 m in this seismic profile (Fig. 3). Similar reflectors were observed in the Orca Basin[10] and the Red Sea brine pools[11].

Assistance from NIOZ, RGD and VML technicians and the Master and crew of the RV *Tyro* together with the help of students from both universities in Amsterdam and invited scientists from the universities of Nice, Milan, Naples and Athens is gratefully acknowledged. X. le Pichon and N. Lyberis kindly supplied the Sea Beam charts. The research cruise was financed by the Netherlands Coucil for Oceanic Research (NRZ).

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Received 26 May; accepted 11 August 1983.

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Recent sapropel formation in the eastern Mediterranean

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During the 1983 expedition of the RV *Tyro* in the eastern Mediterranean, a combined programme of seismological, micropalaeontological and geochemical research was performed. The geochemical research focused mainly on the interstitial water and the chemical composition and origin of sapropels. For this purpose box-cores, piston-cores and bottom-water samples were collected. We report here that some of these cores showed clear evidence of long-term stagnant bottom-water conditions in a small basin south of Crete (for which we propose the name Tyro Basin)[1]. The bottom water is strongly anoxic, whereas the sediment down to at least 400 cm is sapropel to sapropelic. As salt concentrations in the bottom water are eight times higher than in normal seawater, the anoxic conditions in this basin are probably due indirectly to dissolution of salt from a nearby diapir.

Sapropels are known to be present throughout the Mediterranean[2,3], but are most in evidence in the eastern part, at depths over 2,000 m (refs 3–5). Many hypotheses have been put forward to explain the occurrence of these sediment layers with a high content of organic matter[3,6]. A generally accepted definition of a sapropel is "a sediment rich in organic matter (>2% organic C) which has been formed under reducing conditions in a stagnant water body"[7]. Supplementary characteristics are: dark colour, microscopically detectable organic matter, and the absence or extreme scarcity of benthic fossils. Sediment rich in organic matter can also be formed by an extremely high supply of organic matter ('gyttja'). As the origin of organic-rich sediment is not always traceable, any sediment containing over 2% organic C is commonly considered to be a sapropel. The term 'sapropelic' is used for a sediment with 0.5–2.0% organic C[7]. As we show below, the bottom water in the Tyro Basin is both stagnant and reducing, so we can speak of a sapropel to sapropelic sediment *sensu stricto*, but of limited lateral extension.

After collection of the cores, samples were taken for pore water extraction, according to our shipboard routine developed in the past few years[8]. Pore water squeezers were handled under a nitrogen atmosphere (<0.005% oxygen) and at *in situ* temperature (13 °C); both conditions are known to be important in the squeezing of sediments[9–13]. The interstitial water samples were divided into three separate portions to be deep-frozen, acidified and for shipboard analyses. Nutrient concentrations and alkalinity were determined immediately—according to a modified automated method—with a Skalar SA-400 Analyzer and an adapted titration, respectively[14]. Oxygen was determined by the Winkler method[14,15], chloride by the Mohr-titration[16] and the alkali metals were determined by atomic absorption spectrometry.

Cores and bottom-water samples from the Tyro Basin (cores 8, 9 and 46) are strongly anoxic, indicating stagnant bottom-water conditions. Water samples taken approximately 100 and 150 cm above the sediment–water interface were completely depleted of oxygen (Table 1) as shown by on-board analysis, and also emitted a strong odour of H_2S. On opening the box-cores and piston-cores, it became evident that anoxic sediment conditions prevailed throughout the sediment column. The dark-coloured sediment with varying organic-carbon content (1.3–3.2% organic C) had only a few faint laminations, while the surface layer had a jelly-like appearance. Ammonium concentrations were extremely high in core 46 and much higher than in nearby cores outside the basin (Table 1, Fig. 1), whereas the *p*H was very low, probably because of the high H_2S and CO_2 concentrations. The salt effect on the *p*H electrode could also have had some influence[17]. Without any doubt, anoxic conditions must have prevailed in the Tyro Basin for quite some time. Assuming an undisturbed sedimentation and taking a relatively high sedimentation rate of 5 cm kyr^{-1}, we conclude that the recovered sediment column of 400 cm of solely sapropel to sapropelic sediment indicates a period of at least 80,000 yr. The chloride concentration of the bottom water in the Tyro Basin (cores 8, 9, 46) is extremely high (Table 1). Since the temperature of the bottom-water samples is as low as in other

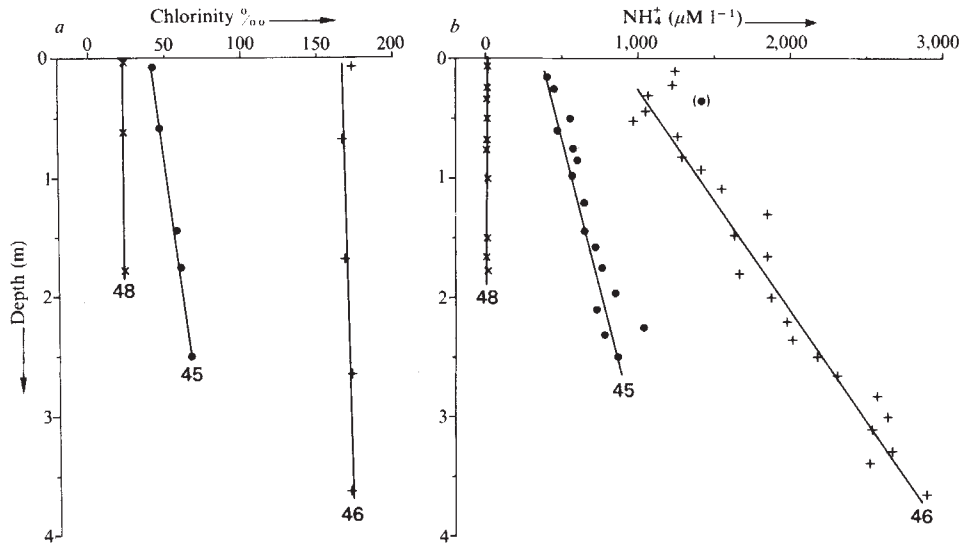

Fig. 1 Chloride (*a*) and ammonium (*b*) concentrations in pore water of cores 45, 46 and 48.

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Table 1 Comparison of the concentration of some elements in the bottom water and upper 50 cm of sediment

Core no.	NH_4^+ concentration ($\mu M\ l^{-1}$) in bottom water	Mean NH_4^+ concentration ($\mu M\ l^{-1}$) in upper 50 cm of core	Oxygen concentration ($mg\ l^{-1}$) in bottom water	Chlorinity ($g\ kg^{-1}$) in bottom water	Mean chlorinity ($g\ kg^{-1}$) in upper 50 cm of core	Mean punch-in *p*H in upper 50 cm of core	Water depth (m)	Position (Loran C) °N	°E
T83-8	750	900	0	162	164	6.5	3,337	33°52.3′	26°01.6′
T83-9	700	800	0	157	167	6.5	3,223	33°52.5′	26°00.7′
T83-46	750	1,200	0	157	172	6.5	3,482	33°52.5′	26°02.3′
T83-45	4	300	6.1	21.6	40	7.7	3,316	33°50.5′	25°56.7′
T83-48	5	6	6.1	21.5	21.5	7.7	2,724	33°50.1′	25°59.1′

Cores 8, 9 and 46 are from the Tyro Basin, core 45 is from an attached basin and core 48 is from a nearby elevation.

Table 2 Comparison of the alkali metals in the Tyro brine with average seawater, Orca brine and Red Sea brine

	Cl($g\ kg^{-1}$)	Na($g\ kg^{-1}$)	Mg($g\ kg^{-1}$)	Ca($g\ kg^{-1}$)	K($g\ kg^{-1}$)
Tyro brine	157	103.5	1.42	1.34	1.30
Average seawater	19.4	10.8	1.29	0.41	0.40
Orca brine	149.5	91.5	1.05	1.09	0.63
Red Sea brine	155.3	92.9	0.81	4.71	2.16

samples outside the basin (12–14 °C), an influx of a hot brine, as in the Red Sea, can be ruled out. Chlorinities of the bottom water in the attached basin (core 45) and on a nearby elevation (core 48) are in accordance with published salinities for this region[18,19]. Because of the hypersaline bottom water there is stratification of the water column over the Tyro Basin. This prevents quick exchange of oxygen-rich water by circulation from surface to bottom. Table 2 compares the concentration of alkali metals in the Tyro brine with that in other brines and average seawater. The brine is almost entirely a halite-solution; compared with the Orca brine[20,21], Mg, Ca and K are relatively enriched. It seems very plausible that a diapir is the possible source of this salt. Salt diapirs of Messinian origin are found in many parts of the Mediterranean[22–24], also close to the Strabo Trench[25,26].

A special case is core 45 (Fig. 1*a*); apparently the bottom-water salinity is at present at a normal level, as is the oxygen concentration[18]. From the pore-water data (Fig. 1*a*, *b*, Table 1), however, it is evident that the same conditions as exist in the Tyro Basin must also once have prevailed here. The large gradient of chlorinity in core 45 (Fig. 1*a*) seems to be diffusion-controlled. Calculations on diffusion can be made, assuming the original chloride concentration to be 200‰ (approximately saturation) and an effective diffusion coefficient of $3\times 10^{-6}\ cm^2\ s^{-1}$. After some simplification, the time that brine water stopped flowing into this basin can be calculated[8,27]. The value found in this way (3,000 yr) is to be considered as an upper limit, as both the salinity and the diffusion coefficient are extreme values, resulting in an overestimated value of time. From that time chlorinity diminished and oxygen slowly returned to the bottom water (Table 1).

The varying organic carbon content in core 46 suggests that sapropel layers should probably be attributed not only to stagnant bottom water but also to a higher production in the surface water. Rossignol-Strick *et al.*[6] proposed a higher than normal run-off from the River Nile to explain stagnation in the eastern Mediterranean. In our view, however, such a large run-off could also have caused a larger than normal input of nutrients into the Mediterranean, which in turn would have caused a higher productivity.

It cannot be ruled out that in the past, local sapropel formation similar to that in the Tyro Basin may also have occurred in other parts of the Mediterranean.

We thank T. Bouten and H. Dekker for carrying out the shipboard analyses and P. R. Alkema, T. Buisman, F. Clotz and J. Schilling for technical assistance. We appreciate the cooperation given by Captain Blok and his crew. Analytical material was kindly put at our disposal by the National Geological Survey (RGD). Financial support was given by the Netherlands Council of Sea Research (NRZ). The expedition of the RV *Tyro* to the eastern Mediterranean is a programme of the Marine Geology group of the Free University and the University of Amsterdam, in conjunction with the Geochemistry group of the Institute of Earth Sciences, State University of Utrecht.

Received 26 May; accepted 11 August 1983.

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Mimesis of bellflower (*Campanula*) by the red helleborine orchid *Cephalanthera rubra*

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The spectacular red helleborine orchid (*Cephalanthera rubra*) is distributed from Iran throughout the Mediterranean region and up to western Europe, reaching southern Scandinavia[1]. The plant preferably grows on calcareous soils in dry woods[2]. The elongate, loose inflorescence usually bears 4–10 brilliantly rose-coloured flowers which completely lack nectar or other food for anthophilous insects and thus act by deceit[3]. Flower-visitors so far reported are bees[4–6], especially males of the solitary bees *Chelostoma fuliginosum* (Pz.) (= *Chelostoma rapunculi* (Lep.) = *Chelostoma nigricornis* (Nyl.)) and *Chelostoma campanularum* (K.) (Megachilidae). Females of the two *Chelostoma* species collect pollen for their brood almost exclusively from bellflowers (*Campanula*)[7]. Mate-seeking flights, feeding and resting of the males are strongly associated with *Campanula* flowers. I now report that *C. rubra* mimics the floral coloration of *Campanula* in the bee visual system and thereby receives pollination service by the *Chelostoma* males. Pollination is regularly effected by males of *C. fuliginosum*. The orchid peaks in anthesis distinctly before *Campanula*, phenologically matching and efficiently exploiting the male bee population.

Interactions between *C. rubra* and anthophilous insects were observed on the island of Gotland in the Baltic Sea during 1980–82. The flowers were visited by various insects but regularly only by males of *C. fuliginosum* and *C. campanularum*. The males performed repetitive patrolling flights[8] between certain 'landmarks' in their habitat. Although *C. rubra* offered no food, they incorporated its flower-spikes into their patrolling routes, repeatedly inspecting and attempting to feed from the flowers. Besides *C. rubra*, these mate-seeking flights regularly included the flowers of *Campanula persicifolia*, the most important food plant for the two *Chelostoma* species in Gotland, and sometimes also *Campanula rotundifolia*. In addition, the males patrolled dry logs, poles and fallen branches (the females construct brood cells in holes adopted from wood-boring insects). When visiting *C. rubra*, the males entered the inner funnel-shaped cavity, formed by the two petals enclosing the column from above and the labellum laterally, as if to explore the flowers for nectar. When males of *C. fuliginosum* attempted to back from this position, they had to depress the distal, hinged portion of the labellum by their abdomen. As a result, the bees stroked firmly against the floral sexual organs and the crescent-shaped pollinia were cemented onto their backs by 'glue' deposited from the stigmatic surface, as formerly suggested for *Cephalanthera longifolia*[9]. The only previous report of a bee carrying pollinia of *C. rubra* was from France, and involved a male of *C. fuliginosum*[6]. Males of *C. campanularum*, because of their smaller size, slipped with ease out of the flowers without removing the pollinia.

Colorimetric analyses of the floral reflectance revealed that in the bee visual spectrum the colours of *C. rubra* and *Campanula* flowers are practically identical (Fig. 1). The colour difference apparent to the human eye is due to the strong reflectance of 600–650 nm (red) in *C. rubra*, which is beyond the range of the bee visual system. Since the primary pollinator of *C. rubra* in its activities is closely associated with *Campanula*, the similarity is interpreted as floral colour mimesis. The flower-lip centrally exhibits conspicuous cream-coloured sinous frilled ribs which might also mimic the sexual organs in *Campanula* flowers.

Chemical analyses by gas chromatography/mass spectrometry[10] showed that the faint floral fragrances of both *C. rubra* and *C. persicifolia* are weak terpene blends, consisting mainly of hydrocarbons. Their constituents are not strikingly similar. Such terpenes are also emitted from the vegetative parts of both species. No observational data suggest chemical attraction of the *Chelostoma* males. Mimesis, therefore, seems to be restricted to coloration. Floral morphology as well as fragrance apparently are of minor importance in this deception system.

At all times except during warm sunny periods, assemblages of *Chelostoma* males were sheltering and resting inside or in the close proximity of *Campanula* flowers. Such males often carried pollinia of *C. rubra*. In a few cases, *C. fuliginosum* males were observed to sit inactive in flowers of *C. rubra*, with only the end of the abdomen visible. However, as the males aggregated exclusively at *Campanula*, shelterings in *C. rubra* were exceptions. Attraction to a shelter or sleeping-hole nevertheless may occasionally contribute to pollination in *C. rubra*. In the orchid genus *Serapias*, in contrast, such attraction occurs on a regular basis[11,12].

The phenology of *C. fuliginosum* females was synchronous with the flowering phenology of *C. persicifolia*. The orchid, however, began flowering and peaked in anthesis much earlier than the *Campanula* population. The difference in flowering phenology was about 2 weeks, and at the peak of *C. rubra* anthesis only about 8% of the *C. persicifolia* population were in flower. As the males of *Chelostoma* emerge from the outer cells in the nest burrow markedly earlier than the females, the comparatively early *C. rubra* population perfectly matched the

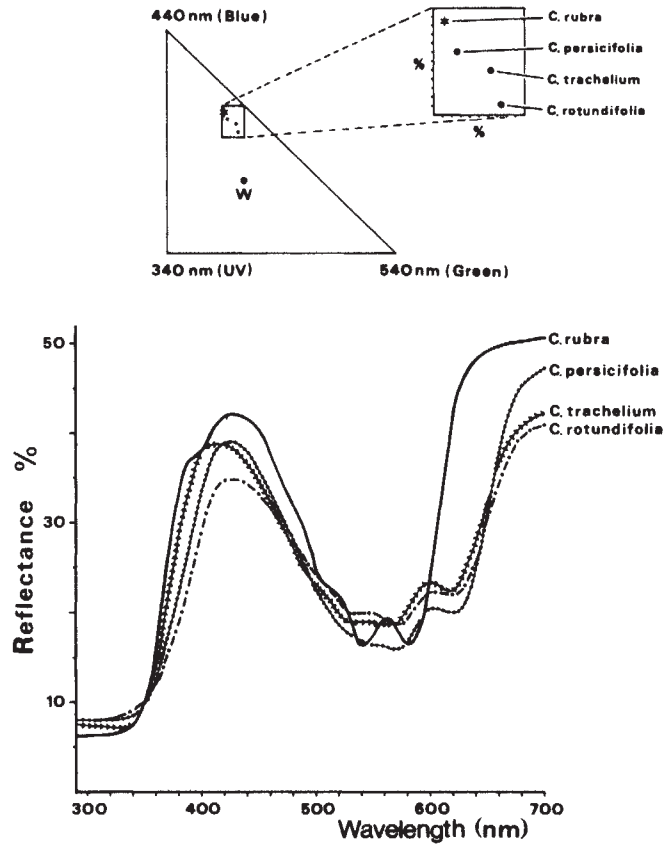

Fig. 1 Spectral reflectance curves of the flowers of *Cephalanthera rubra* and three *Campanula* species (bottom). Note the close similarity between the colour of mimet (orchid) and model (*Campanula*) also as trichromatic plots in the chromaticity diagram[17] based on the bee visual spectrum[18] (top). W is the equiproportionate reflectance white point. Reflectance curves were obtained by a Perkin–Elmer 554 UV/visible spectrophotometer with internal integrating sphere attachment.

0028–0836/83/430799—02$01.00

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Table 1 Fruit set in *C. rubra* at different sites in relation to occurrence of patrolling *C. fuliginosum* males

	Patches of *C. rubra*	Mean fruit set (%)	s.d.	No. of plants
I	In intensely patrolled area*	30.1	23.3	41
II	In virtually unpatrolled areas (distance from I indicated in parentheses)			
	Under pines (5 m)	7.0†	6.5	5
	Under pines (E 50 m)	2.9‡	5.8	32
	In pine wood (N 75 m)	5.1‡	10.6	51
	On hillock (NE 100 m)	5.9‡	13.0	60
	On hillock (E 150 m)	0‡		11

Fruit set is number of fruits/number of flowers per plant. Data were taken from Irevik, Gotland, in 1982.

* In sympatry with *C. persicifolia* and nest sites of *C. fuliginosum* in old fences, telegraph poles, etc.

† Significantly different from patrolled patch. Wilcoxon two-sample test, $P<0.05$; ‡ $P<0.001$.

phenology of the male population. So far, female *Chelostoma* have not been recorded as visitors to *C. rubra*. Outside those areas heavily patrolled by *C. fuliginosum* males, the reproductive success of *C. rubra* in terms of fruit production was drastically reduced (Table 1). A sixfold difference in fruit set was recorded between patrolled versus virtually unpatrolled subhabitats. Stray males infrequently contributed to pollination outside the patrolled rendezvous area. Males of *Chelostoma* normally do not fly far from the nest area, usually less than 60 m (ref. 13). In places where *C. fuliginosum* was absent, populations of *C. rubra* did not set any fruit. Abundance of *C. rubra* also outside the patrolled area did not cause the male bee population to 'disperse' among the *C. rubra* individuals and to start scattered patrolling far out from their home range. If this were to happen, males probably would have had less probability of encountering virgin females. Hence, *C. rubra* should be considered as a harmless 'hanger-on' to the relationship between *Chelostoma* and *Campanula*. Locally, certain orchid-spikes might even assist in keeping together the population of male bees in subhabitats where virgin females emerge and mate. Later the females pollinate *Campanula* during their pollen-collecting bouts.

The cream-coloured ribs on the flower-lip of *C. rubra* are yellow-crested and smooth on top. Its relative, *C. longifolia*, exhibits certain detachable papillae, 'pseudo-pollen', on the labellum ridges and deceives pollen-collecting female bees of the genus *Halictus* s.l.[14,15]. *C. longifolia* is said to mimic *Cistus salviifolius*, although the two plants differ completely in their floral UV reflectance as well as distribution[15]. In the relationship between *C. rubra* and *Campanula*, both model and mimet have similar reflectance over the range of bee vision. Furthermore, the distribution of *C. rubra* in Europe lies within (and coincides well with) that of the common blue-flowered *Campanula* species, for example, *C. persicifolia*[16]. The pollination system of *C. rubra* is dependent on several factors including sympatry of *C. fuliginosum*, sympatry of *Campanula*, presence of dry wood with bore-holes and occurrence of wood-boring insects (for example, longicorn beetles).

This work was supported by the Swedish Natural Science Research Council. I thank I. Groth and I. Johansson for assistance in the fragrance and reflectance analyses, respectively, and B. Kullenberg, S. Ulfstrand and A. Nilsson for comments on the manuscript.

Received 7 June; accepted 2 September 1983.

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Unified response to bilateral hemispheric stimulation by a split-brain patient

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Surgical sectioning of the corpus callosum in epileptic patients has provided a unique opportunity to study separately the competence and processing capacities of the two cerebral hemispheres, each of which is able to perceive, think, memorize and learn independently and essentially outside the realm of awareness of the other[1–4]. While research has focused on this 'disconnection syndrome', split-brain patients nonetheless behave as unified individuals in their normal environment, and the present study investigated this aspect of their behaviour in an experimental setting. The two hemispheres of a callosotomized patient were simultaneously presented with information associated with conflicting responses, and the subject was requested to produce a single response. In all combinations of hemisphere stimulation and hand responding, the subject was capable of perfect accuracy, suggesting that he could integrate and resolve the conflicting information before the production of his response, and that his two disconnected hemispheres were simultaneously aiming at the same goal.

One startling fact about commissurotomized patients is that, despite having two independent and different cognitive processors, they behave as unified individuals and seldom display signs of hesitation, confusion or dissociation in their day-to-day activities[3,4]. Were it not for results from specially designed experiments, cutting the corpus callosum would be regarded as resulting in no neurological or psychological sequelae. One explanation for this apparent absence of hemisphere disconnection syndrome outside experimental situations has been the suggestion that the same information is projected to both hemispheres through, for instance, head and eye movements, whereas in laboratory settings information can be directed to only one hemisphere by using the anatomical property of the visual system whereby the right and left visual fields are represented separately in the left and right hemisphere, respectively[4]. However, such an explanation does not resolve the contradiction, because different decisions may be reached by each hemisphere after processing the same information. For example, the right hemisphere of a split-brain patient matches pictures according to visual similarity whereas the left hemisphere matches the same pictures according to functional association; this results in qualitatively different responses from the two hemispheres[5].

Thus, while casual observation of commissurotomized patients indicate that they are capable of, and indeed display, unified purposeful behaviour, there is little experimental evidence that they can attend simultaneously to both fields of stimulation and integrate the incoming information received by each hemisphere. In fact, experiments with split-brain patients have mainly consisted of either a unilateral presentation followed by one

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response from the stimulated hemisphere, or bilateral simultaneous presentation followed by two responses, one from each stimulated hemisphere. In the latter condition, double responses to stimuli in both fields are extremely rare, and patients generally respond to one field, ignoring the other[6]. All the above experimental situations were deliberately framed so as to encourage the two hemispheres to operate in isolation from each other, right through from stimulus reception to response production, and they were unsuitable for addressing the issue of possible communication between the two hemispheres. To investigate the capacity of a split-brain patient to integrate the information received by the two hemispheres, an experiment was designed involving simultaneous bilateral presentation and requiring the subject to make a single response.

The subject was J.W., a 28-yr-old right-handed man, operated on in two stages by D. H. Wilson in 1979, and already tested by Gazzaniga and his colleagues pre-, inter- and post-operatively[7–9]. J.W. displays some language comprehension but no production in his right hemisphere, and, although his anterior commissure was left intact, he has shown the typical disconnection syndrome.

The task was letter-categorization in which J.W. was to decide whether or not a letter was a vowel. The stimuli were 8 letters, 4 vowels and 4 consonants, and they were flashed 2° to the right or left of fixation for 100 ms on a video screen of an Apple II computer. Two Morse keys were placed in front of J.W., and he was to press one key if a vowel was presented and the other key if no vowel was presented. J.W. responded by moving one arm from a central resting position to one of the two keys, and the right arm was always tested first. Response latency and accuracy were automatically recorded.

A preliminary experiment consisted of a unilateral presentation of one letter, and the task was to press one key or the other depending on the category of the letter. This condition was run in two 48-trial sessions, each one with a different hand, and each session was preceded by 12 practice trials. The aim of this condition was to establish the skill of each hemisphere to perform the letter-categorization task. The results (shown in Table 1) indicated that the two hemispheres were equally competent in deciding whether or not a letter was a vowel, in terms of both speed and accuracy. The finding therefore provided a guarantee that any response asymmetry in the subsequent bilateral presentation condition could not be attributed to a differential hemispheric competence in performing this letter categorization task.

In the main experiment of this investigation, two letters were simultaneously flashed so that one letter was projected to the right hemisphere and the other to the left. Six different combinations of the two-letter presentation were possible: two identical vowels, two different vowels, a vowel to the right hemisphere and a consonant to the left, a vowel to the left hemisphere and a consonant to the right, two identical consonants, and two different consonants. J.W. was requested to press one key if at least one vowel was presented and to press the other key if no vowel was presented. Two 96-trial sessions were run, each one with a different responding hand, and 12 practice trials preceded the experimental sessions. The two important letter combinations were those in which different categories of letter were projected to each hemisphere, resulting in conflicting information to be processed before making a correct decision. Current understanding of split-brain functioning would suggest that the conflict condition should give rise either to one letter being ignored, presumably that in the visual field contralateral to the responding hand, or to one hemisphere taking over, presumably the left in this language-related task, or to response confusion and hesitation due to opposite decision reached by the two hemispheres[3,4].

The results (shown in Table 2) did not conform to any of these predictions. J.W. displayed no overt signs of hesitation throughout the experiment, and he was capable of perfectly accurate performance with either hand in the conflict condition, suggesting that the different decisions made by each hemisphere could somehow be resolved before a voluntary response was executed. Even though the hand directly controlled by the hemisphere receiving the vowel responded faster than the other hand, this did not result in one hemisphere taking over nor in one field being ignored. In addition, while latencies to the presentation of two vowels were shorter than in the conflict conditions, reaction times to the conflict conditions were no longer than those to the presentation of two consonants, suggesting that little, if any, confusion took place when conflicting information was presented to the two hemispheres. This is evidence that sectioning the corpus callosum may not prevent all forms of communication between the two hemispheres nor preclude integrated voluntary behaviour.

An additional experiment was then done to examine on what basis interhemispheric communication could be achieved. Two

Table 1 Unilateral presentation in letter-categorization tests in a split-brain patient

		Vowel		Non-vowel	
		LVF-RH	RVF-LH	LVF-RH	RVF-LH
Right hand	Mean (ms)	564	538	640	676
	s.e.	56	43	29	51
	% Correct	92	100	100	100
Left hand	Mean (ms)	578	619	702	731
	s.e.	32	24	29	35
	% Correct	100	100	100	100

In the preliminary experiment, a vowel or a consonant was flashed either in the left visual field which projects to the right hemisphere ('LVF-RH') or in the right visual field which projects to the left hemisphere ('RVF-LH'). The table gives the mean latencies (in ms), standard errors and percentages of correct responses, to decide whether the unilaterally presented letter was a vowel or was not a vowel, as a function of the responding hand.

Table 2 Bilateral presentation in letter-categorization tests in a split-brain patient

		Same vowels	Diff. vowels	Vow.-RH Cons.-LH	Vow.-LH Cons.-RH	Same cons.	Diff. cons.
Right hand	Mean (ms)	574	556	771	739	843	725
	s.e.	14	25	63	53	67	42
	% Correct	100	100	100	100	92	75
Left hand	Mean (ms)	466	442	563	793	713	687
	s.e.	21	15	33	51	42	59
	% Correct	92	100	100	100	83	100

In the main experiment, two letters were simultaneously flashed, one in each visual field. The two letters could be either the same vowels, different vowels ('Diff. vowels'), a vowel to the right hemisphere and a consonant to the left hemisphere ('Vow.-RH, Cons.-LH'), a vowel to the left hemisphere and a consonant to the right hemisphere ('Vow.-LH, Cons.-RH'), the same consontants or different consonants ('Diff. cons.'). The table gives the mean latencies (in ms), the standard errors and the percentage of correct responses for each of the six letter combinations, as a function of the responding hand.

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letters were again simultaneously presented, one in each visual field, but this time J.W. was requested to press one key if the two letters were identical and the other key if they were different, irrespective of their category. In all other respects, this experiment was similar to the main experiment, using the same letter combinations, which implied an unequal number of identical-letter and different-letter trials (24 and 72, respectively). J.W. proved unable to match the bilaterally presented letters, and his performance was not significantly different from chance: he responded correctly in 58% of identical-letter trials and in 43% of different-letter trials, for an overall accuracy of 47% (in all cases, $P > 0.35$). Response latencies were longer than in the main experiment and averaged 1,143 ms (s.e. 119); latencies were of the same order in identical-letter and different-letter trials (1,174 and 1,112 ms, respectively). These results are typical of the disconnection syndrome frequently described in split-brain research[3,4].

J.W.'s responses were further analysed to examine whether he had kept responding according to letter category, as in the main experiment, but this was not the case, and he apparently responded at random. This indicates that the identity of the letter was known only to the hemisphere that received it, and that the anterior commissure could not convey this information across the hemispheres. It also suggests that interhemispheric communication observed in the main experiment could be achieved only after a decision on the category of the letter had been made within each hemisphere. Information about the outcome of such independent decisions may thus be available to some (subcortical) structure to which each hemisphere is linked and in which some integration can be achieved, at least when a limited number of alternatives are involved.

When simultaneously presented with conflicting information in a task at which the two hemispheres are equally competent, a split-brain patient can attend to both fields of presentation and resolve the contradictory decision made within each hemisphere. This performance was achieved with either hand and when either hemisphere received the information associated with the response, but without transfer of the identity of the letter projected to each hemisphere. This finding confirms previous suggestions[10] that subcortical structures may provide a link between the two disconnected hemispheres, as recently illustrated by the priming of one hemisphere by information received by the other[9,11], and it further indicates a capacity by split-brain patients to integrate conflicting information and to display unified purposeful behaviour. This is consistent with the recent suggestion[12] that "at the higher levels of supervisory evaluation J.W. has not two half-systems but one undivided system, presumably associated with deep neural structures not surgically separated". Whereas much speculation has surrounded the interpretation of findings from research on commissurotomized patients, the present result stands against equating 'split-brain' with 'split-mind', and suggests that the human brain may be striving to maintain mental unity.

I thank Michael Gazzaniga and Jeffrey Holtzman for allowing me to test J.W., and for their support and suggestions, and Michael Corballis, Peter Milner and Harry Whitaker for their comments and suggestions. During this work, I was partially supported by the Stairs Fund of McGill University.

Received 18 March; accepted 15 August 1983.

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Field effects trigger post-anodal rebound excitation in vertebrate CNS

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Although both chemical and electrotonic synaptic interactions have often been implicated in normal and pathophysiological conditions where clusters of central neurones discharge in unison[1–5], in many instances the mechanisms underlying synchrony in the vertebrate central nervous system remain obscure. Another form of cellular communication which can be invoked is that of field effects[3], defined here as electrical interactions mediated across extracellular space. Studies of the goldfish Mauthner (M-) cell provided the first clear evidence for such interactions, which have now been shown to exist in various mammalian central structures[6–10]. A single impulse in the M-cell leads to the nearly simultaneous firing of 40 to 80 interneurones which feed inhibition back onto it[11,12], by both direct synaptic excitation and a field effect, which is initially hyperpolarizing. On the basis of early observations on this system, it was suggested that field effects could induce or facilitate synchronized and even epileptic-like neuronal bursting[13]. We report here that the inhibitory interneurones exhibit a remarkably sensitive anodal break excitation triggered by a brief hyperpolarization comparable to the electrical inhibition mentioned above. This mechanism alone may be sufficient to recruit a second class of these interneurones[14,15], which are postsynaptic to eighth nerve afferents and do not receive chemical synaptic input from the collateral network. The rebound facilitation is distinguished from excitatory field effects which can also contribute to synchronization.

Experiments were conducted with goldfish (*Carassius auratus*) anaesthetized initially with MS-222, immobilized with (+)tubocurarine and respirated with tap water circulated over the gills. Intracellular recordings were obtained from the interneurones (Fig. 1*A*) known to mediate electrical and chemical inhibition of the M-cell[11,16,17] using KCl- or K_2SO_4-filled micropipettes. The data reported here are from cells having resting potentials in the range of −50 to −65 mV and spike potentials of at least 75 mV. These neurons are all identifiable by a passive hyperpolarizing potential (p.h.p), which is a brief (0.5 to 1.0 ms) field effect hyperpolarization[11,18] directly produced by the large extracellular currents associated with the M-cell action potential (Fig. 1*B*). This p.h.p. is typically a few millivolts in amplitude, occurs simultaneously in many interneurones, and precedes their synchronous activation underlying the recurrent inhibition. As shown in Fig. 1*B* the synchrony is signalled by an extracellular positivity in the axon cap[12], that is, in the neuropil surrounding the M-cell's axon hillock and initial segment (region surrounded by dashed line in Fig. 1*A*). As described previously[13], this positive field both reflects and contributes to the synchronization of the interneurones, as its underlying current provides a means by which these cells are mutually excitatory. That is, the currents produced by active p.h.p. cells will further depolarize adjacent neurones which have not yet reached threshold. This phenomenon is superimposed upon the other facilitatory influences and is dependent on ongoing excitation of the interneurones (see below).

Intracellular current injections were used to produce hyperpolarizations of the interneurones comparable to p.h.ps. These hyperpolarizations were often followed by an anodal break

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increase in excitability seen either as a reduced threshold for excitation by a test depolarizing current pulse or, in the majority of the cells studied, as a frank depolarization which could lead to spike initiation; only the latter will be illustrated in this communication. In Fig. 1C_1, the rebound depolarization is similar in amplitude but longer lasting than the 3.0 mV potential which triggered it. When the stimulus duration was increased to ~2.0 ms, the depolarization at pulse offset was sufficient to reach threshold for spike initiation (Fig. 1C_2). These striking post-inhibitory rebounds are likely to be of functional significance, as they can summate with subthreshold excitatory postsynaptic potentials to produce orthodromic impulses (Fig. 1D). The time course of the anodal break excitation is also consistent with the idea that it contributes to synchrony of the p.h.p. cells. The peak times of the rebound depolarizations and the latencies of anodal break spikes were typically in the range of 1.0 to 2.0 ms after pulse offset, which is also the latency range for the impulse discharges evoked during recurrent inhibition of the M-cell.

To investigate the mechanisms underlying the generation of this anodal break excitation, we have studied the effects of varying both amplitude and duration of the conditioning hyperpolarization. One significant observation is that with slightly prolonged constant current pulses (greater than a few milliseconds duration), the hyperpolarization itself may decay markedly (Fig. 2A), suggesting that there is a time-dependent inward rectification[19,20]. In some cells, this decay was most pronounced for moderate hyperpolarizations and was decreased or abolished if the membrane potential was hyperpolarized more than 10 mV for 10 to 20 ms; in these conditions the anodal break depolarization could still be demonstrated, although it was minimal and had a delayed onset. Thus, the anodal break phenomena are triggered by short-lasting hyperpolarizations comparable in amplitude to the p.h.ps. evoked by physiological stimuli. As illustrated in Fig. 2B_1 and B_2, when the moderate stimulations were used the magnitude of the depolarization at pulse offset was enhanced by increasing the duration (Fig. 2B_1) and/or magnitude (Fig. 2B_2) of the conditioning hyperpolarization. Within a range of hyperpolarizing stimuli of up to about 10 mV, there was a linear relation between the evoked anodal break depolarization and the product of pulse duration times the peak hyperpolarization (Fig. 2C). Since the peak hyperpolarization is directly proportional to the applied current (see also ref. 18), the anodal depolarization is, therefore, a linear function of the time integral of that current.

Anodal break excitation and the time-dependent inward rectification which develops during applied hyperpolarizations have been studied in a number of cell types, including motoneurones and dorsal root ganglion cells[19,21–23]. Mechanisms postulated to explain these phenomena include delayed changes in potassium, chloride or sodium conductances appropriate for a decrease in the hyperpolarization and/or a lowered threshold at the pulse offset. The extreme sensitivity of the postanodal excitation reported here, however, seems to rule out several of the potential mechanisms, since they either become manifest at levels of membrane hyperpolarization beyond those studied in this case (for example, anomalous rectification[20] and the Q current described for hippocampal neurones[24]) or have rate constants which are clearly too slow (for example, deactivation of the potassium-dependent M-current during hyperpolarization[24,25]). The rapid time course of the anodal break excitation described here suggests that for these interneurones the most significant factor is likely to be a time-dependent change in sodium conductance. Specifically, we propose that at the observed resting potential there is a significant sodium inactivation;

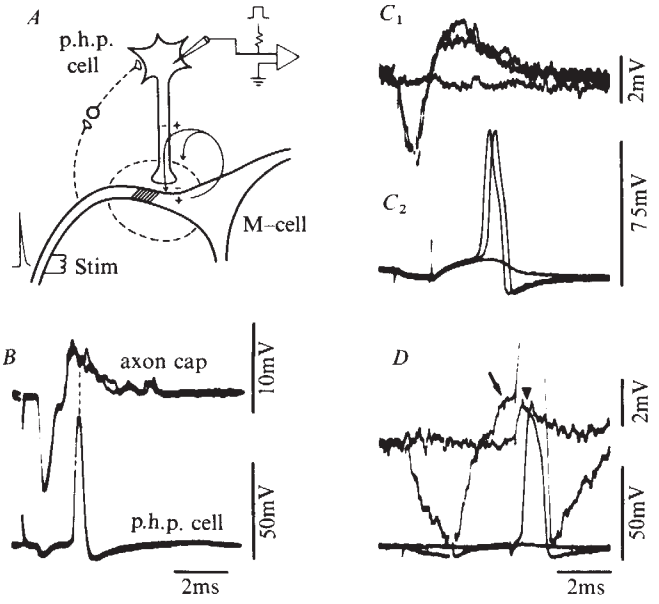

Fig. 1 Anodal break depolarizations of inhibitory interneurones. A, Diagram of the experimental set-up, indicating that intracellular recordings were obtained from identified p.h.p. cells, some of which are activated by the Mauthner (M-) cell's recurrent collateral network (dashed disynaptic pathway). Antidromic activation (stim) of the M-axon was used for identification of the interneurones. The action potential generates an extracellular negativity in the axon cap (region enclosed by dashed line) and hyperpolarizes the p.h.p. cell processes. B, Simultaneous recordings from the axon cap and a p.h.p. cell; the latter fires during the extracellular positivity, which signals synchronous excitation of 40 to 80 such neurones. C_1, C_2, D: Anodal break excitation of two different p.h.p. cells. In C_1 and C_2, hyperpolarizing current pulses of 0.7 and 1.7 ms durations, respectively, evoked sub-threshold and threshold depolarizations of the neuron. In D, a subthreshold excitatory postsynaptic potential (arrowhead) evoked by stimulation of the ipsilateral eighth nerve summated with the anodal break depolarization (arrow) to initiate an impulse. Upper and lower records are high and low gain, respectively. Superimposed sweeps with and without the conditioning hyperpolarization; stimulus artifacts have been retouched to establish the continuity of the individual traces. In this and in Fig. 2, constant current pulses were used to produce the hyperpolarizations.

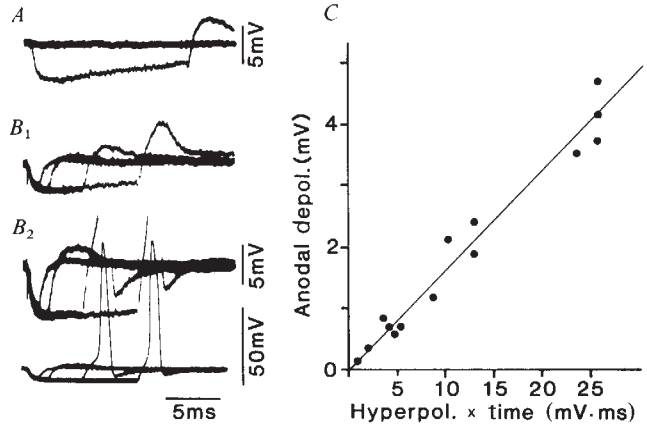

Fig. 2 Time and voltage dependence of the anodal break depolarization. A, With a long current pulse the conditioning hyperpolarization decreases steadily and there is a frank depolarization at pulse offset. B_1, B_2: data obtained from another p.h.p. cell, indicating that the magnitude of the anodal break depolarization depends upon both duration and magnitude of the conditioning hyperpolarization. Peak hyperpolarizations in B_1 and B_2 were 3.0 and 6.2 mV, respectively. The longer duration stimuli evoked impulses in B_2 but only produced subthreshold depolarizations in B_1. In each case superimposed traces illustrate responses to fixed amplitude stimuli of varying duration. All records are d.c. coupled and in B_2 high and low gain recordings are presented. C, Plot of the peak anodal depolarization, measured with respect to resting membrane potential, versus the product of the peak hyperpolarization and stimulus duration. Data are taken from the cell of B_1 and B_2 and responses at or near threshold (~30 mV·ms) are not included. Plotted also is the linear regression line, $y = 0.16x + 0.02$, ($r^2 = 0.96$).

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the partial removal of this inactivation during membrane hyperpolarization could lead to an increased inward sodium current, assuming there is an appreciable level of sodium activation at membrane potentials of −50 to −60 mV. In addition, a persistent non-inactivating sodium current, such as has been shown for cat spinal motoneurones[26], could contribute to the rebound excitation. This hypothesis, which most likely requires that there be two different kinds of Na^+ channels, can also explain the observation that the rebound is often depressed by large and/or long anodal polarizations: the greater hyperpolarization would again partially remove sodium inactivation, but it might also lower the level of sodium activation appreciably, such that any net increase in sodium conductance would be delayed and reduced.

Post-inhibitory facilitations have been studied since the early discussions of Sherrington[27], and rebound excitations following hyperpolarizing inhibitory postsynaptic potentials[28,29] have also been noted in a number of cell types, including those in central structures[30] which exhibit rhythmic synchronized activity. The M-cell system, with identifiable interneurones having a high susceptibility to postanodal excitation, provides useful insights into these phenomena and into the various types of networks where field effects are capable of effecting neuronal synchronization. The term 'field effects' refers to a variety of situations where the extracellular currents generated by one or many nerve cells alter the excitability of others by being channelled across the membranes of the latter. Examples include ephaptic interactions between adjacent nerve fibres, whether occurring naturally[31,32], pathologically[33], or by experimental manipulation[34], facilitated antidromic invasion of spinal motoneurones stimulated simultaneously[6], and the electrical inhibitions described for the Mauthner cell system[11,12] and for the rat cerebellar cortex[7]. Although in some instances there may be specializations of the neuronal tissue, such as the M-cell's axon cap[11], which enhance the field effects, these are all examples of the same fundamental mechanism. Thus, the evidence presented here and previous findings allow us to construct the simple model which is shown in Fig. 3 and accounts for the different mechanisms by which field effects may act alone or in concert with synaptic influences. To summarize the relevant mechanisms, three synchronously activated neurones are illustrated. The first cell reacts to an inhibitory field effect with a threshold postanodal excitation, while the second, which represents a more common situation, fires when this excitatory influence summates with a sub-threshold excitatory postsynaptic potential. Thus, electrical inhibition triggers an active response which alone or together with synaptic input is sufficient to initiate time-locked firing. That is, the excitatory postsynaptic potentials and the post-inhibitory rebound are driving forces underlying synchrony. The third cell shown schematically in Fig. 3 demonstrates that a depolarizing field effect may enhance this behaviour. In this example, the field effect hyperpolarization is ignored, to indicate that since the interneurones have similar orientations, passive depolarizing potentials can be produced by the action currents of adjacent cells. Consequently, the cells are weakly 'coupled' through the extracellular space, and the field effect depolarizations serve to strengthen an emerging or ongoing synchrony by recruiting additional neurones.

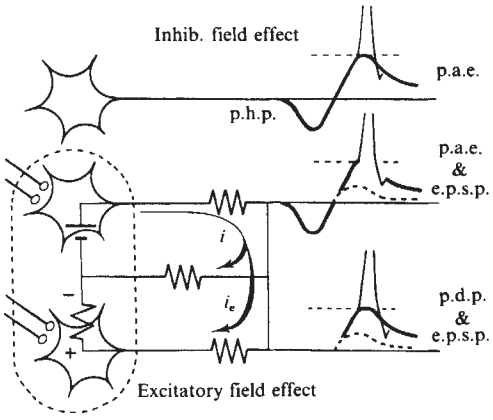

Fig. 3 Hyperpolarizing and depolarizing field effects and their contributions to neuronal synchrony. Diagrammatic representation of three cells in parallel. The upper two are subjected to an inhibitory field effect (p.h.p.) which is followed by a post anodal excitation (p.a.e.). The post anodal rebound can either reach threshold (dashed horizontal line), thus firing the cell (upper neurone), or contribute to synchronization by summating with an otherwise subthreshold exitatory postsynaptic potential (e.p.s.p.) (middle cell). The electrical network connecting the lower two cells illustrates the generation of excitatory field effects due to the action currents produced by a spike in one of them. The current, *i*, splits into two components, returning to the generator (battery) through an extracellular pathway (intermediate resistance) and an intracellular one, where it is excitatory (i_e) at the spike initiating site (depolarization of that region is indicated by the +, − signs). In the lower example the resultant passive depolarizing potential (p.d.p.) acts in conjunction with a subthreshold e.p.s.p., although it could also fire the cell alone (not shown). The dashed oval encompassing the lower two cells indicates that they represent a pool of mutually excitatory interneurones coupled through the extracellular space. For simplicity, each resting membrane potential has been superimposed on an extended neurite. Inputs producing subthreshold e.p.s.p.s (dashed responses) are shown to the left. Potentials related to the field effects are presented as thick lines.

In all cases depicted in Fig. 3, a critical component of the neuronal synchronization is the field potential, which may be generated by the extracellular currents of one neurone, such as the M-cell, or of a population of cells firing together. For example, in the mammalian nervous system, the tendency of nerve fibres lying in parallel to fire together under normal or dystrophic conditions[33], the neuronal synchrony and spread of epileptic foci, occurring possibly in conjunction with glial scarring and an occluded extracellular space, and the electrical inhibition of cerebellar Purkinje cells[7], which is presumably related to the specialized junctions around those neurones, all seem to involve field effects generated by groups of neurones. It now seems appropriate to determine the extent to which this mechanism is complemented in such instances by individual cellular membrane properties or by network organization.

This work was supported in part by NIH grant NS 15335. We thank J. Jordan and J. Seiler for secretarial assistance and J. Lakatos for preparing the figures.

Received 13 May; accepted 9 August 1983.

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Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA

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In the mammalian central nervous system, glycine and γ-aminobutyric acid (GABA) bind to specific and distinct receptors[1–4] and cause an increase in membrane conductance to Cl^- (refs 5–7). Neurones in various regions of the nervous system show differential sensitivity to glycine and GABA[2,3]; thus GABA and glycine receptors are spatially distinct from one another. However, on the basis of desensitization experiments on spinal cord neurones, it was suggested that the receptors for glycine and GABA may share the same Cl^- channel[8]. We now report that in small membrane patches, isolated from the soma of spinal neurones, both receptor channels display several (multiple) conductance states. Two of the states are common to both receptor channels. However, the most frequently observed 'main conductance states' of the GABA and glycine receptor channels are different. Both channels display the same anion selectivity. We propose that one class of multistate Cl^-channel is coupled to either GABA or glycine receptors. The main conductance state adopted by this channel is determined by the receptor to which it is coupled.

Experiments were performed on cultured spinal cord neurones prepared from 13-day mouse embryos[9]. Neurones were kept in culture for 4–12 weeks before the experiment. Single-channel currents were recorded from isolated, outside-out membrane patches[10,11]. In most patches in the absence of either GABA or glycine, apparently 'spontaneous' inward currents are observed in normal bath solution at negative membrane potentials. One class represents openings of a cation-selective channel that is blocked by perfusion of the bath with a solution containing $Tris^+$ as the predominant cation. Another class represents openings of Cl^--selective channels. High-Tris solution does not alter their current amplitude. They display conductance and pharmacological properties similar to the agonist-activated Cl^- channels described below. We believe they are activated by inhibitory transmitter released from nerve terminals still attached to the outside-out patch. During exchange of the bath solution after patch isolation, these Cl^- currents show a time-dependent decrease in frequency of occurrence which is complete on many patches after 5 min. With this procedure outside-out membrane patches that displayed no spontaneous currents are obtained.

Figure 1 illustrates the different types of current response to sequential applications of GABA and glycine in three patches isolated from different neurones and held at −70 mV membrane potential. In the patch described in Fig. 1*a* application of 50 μM GABA did not increase the membrane current, indicating the absence of GABA receptor channels. Application of 50 μM glycine, however, increased membrane current by superposition of several unitary current steps of ~3 pA in amplitude. The patch illustrated in Fig. 1*b* was insensitive to application of glycine, but application of 20 μM GABA caused current steps of ~2 pA in amplitude. For the patch described in Fig. 1*c*, initial application of 5 μM GABA activated 2 pA current steps at a low frequency. When the GABA concentration was increased to 50 μM, the number of activated channels increased initially but then the current became rapidly (<90 s) and completely desensitized. The addition of 20 μM glycine approximately 2 min after GABA application, but with GABA still present, activated current steps of ~3 pA in amplitude (Fig. 1*c*, lower trace). Patches isolated from the same neurone also differed in their sensitivity to GABA and glycine: of the 40 patches tested, 15 responded to both GABA and glycine, 11 responded to only glycine, 8 to only GABA and 6 were insensitive to both transmitters. These experiments show that GABA and glycine receptor

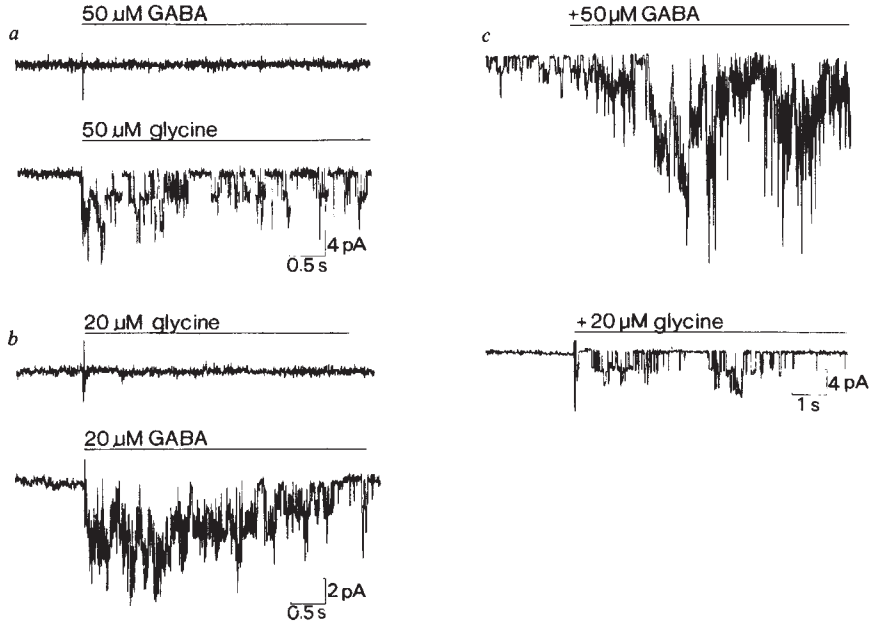

Fig. 1 Glycine and GABA receptor channel currents recorded from outside-out membrane patches isolated from the soma of three different spinal cord neurones. Patches were isolated from neurones bathed in normal bath solution (in mM: 140 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 1 KCl and 10 Na-HEPES, *p*H 7.2), which was then exchanged for one containing $Tris^+$ as the major cation. For most patches this procedure removed background current activity. $Tris^+$ substitution did not alter either the chemosensitivity of the membrane to GABA or to glycine or the conductance properties of the activated Cl^- channels. The pipette solution facing the intracellular side was (in mM) 140 KCl, 3 NaCl, 1 $MgCl_2$, 11 K-EGTA and 10 K-HEPES, (*p*H 7.2). All recordings were carried out at room temperature (22–25 °C). *a*, The top trace shows the absence of current activity before and after rapid application (indicated by the bar) of 50 μM GABA to the bath solution. GABA was then washed out and the lower trace shows the increase in current, caused by the addition of 50 μM glycine (day 35 neurone). *b*, Outside-out patch in which application of 20 μM glycine caused no increase in current but following washout of glycine and application of 20 μM GABA there was a large increase in the current which decreased with time so that current steps of ~2 pA were discernible. Note the much noisier appearance of the GABA-activated current compared with the glycine-activated current in *a*, which reflects the higher frequency of brief interruptions evident in single GABA receptor currents compared with glycine receptor currents. For example, compare the lower traces in Fig. 2*a* and *b* (day 42 neurone). *c*, Outside-out patch in which application of 5 μM GABA (not shown) activated a relatively low frequency of current steps (≃2 pA in amplitude), but when the GABA concentration was increased (indicated by the bar) to 50 μM, additional channels were activated. At this higher concentration the current response quickly and completely desensitized so that after 90 s, no current steps were evident. At this point, addition of 20 μM glycine to the bath solution activated current steps of ~3 pA in amplitude.

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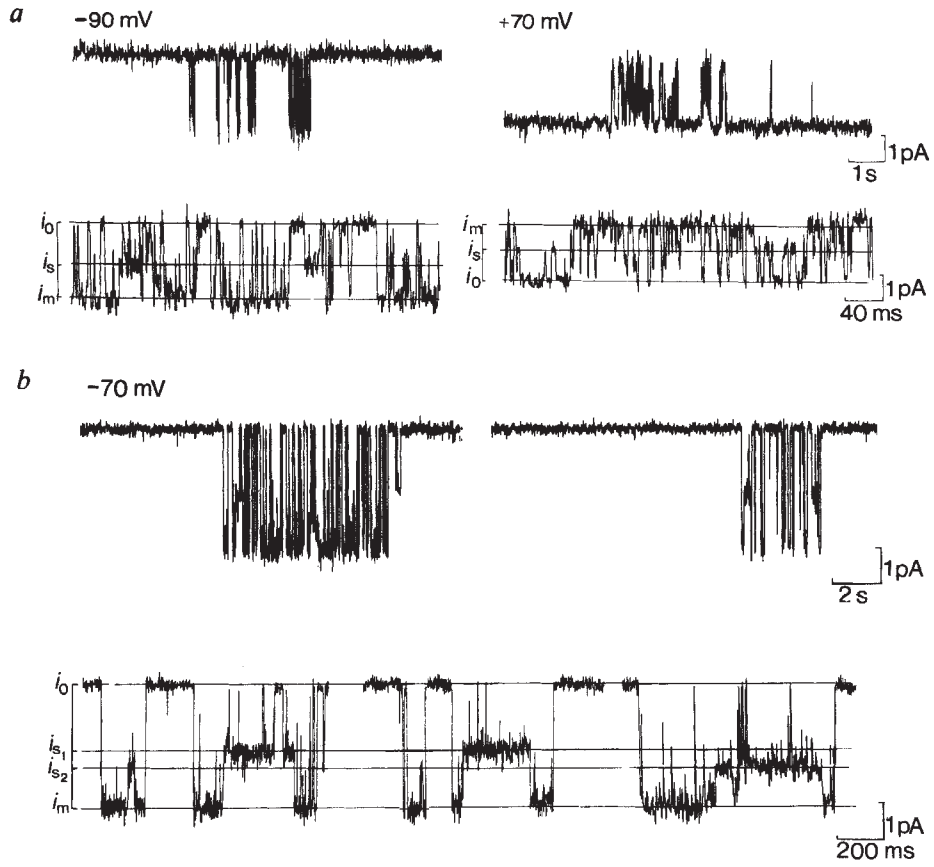

Fig. 2 Bursts of GABA and glycine receptor channel currents recorded from different patches activated by desensitizing concentrations of *a*, 20 μM GABA and *b*, 50 μM glycine respectively. *a*, Current bursts recorded at −90 mV and +70 mV from an outside-out patch following partial desensitization of the current. Internal K^+ was replaced by $Tris^+$ to allow Cl^- channels to be studied in isolation from K^+ channels that are activated by membrane depolarization. The pipette solution was (in mM) 140 Tris-Cl, 3 NaCl, 1 $MgCl_2$, 11 EGTA and 10 Na-HEPES, *p*H 7.2. The initial increase in current caused by 20 μM GABA gradually decreased and returned to the resting level as channels dropped out due to desensitization. Then bursts of current lasting ~0.2–2 s which occurred in clusters of 2–10 bursts could be recorded (upper traces). Bursts, which are separated by long silent periods of up to 20 s, probably reflect the open–closed transitions of individual channels as they transiently leave the desensitized state[14]. Examination of events within bursts at higher resolution (lower traces) indicated brief openings of 1–10 ms that were interrupted by brief (<2 ms) closing gaps. Two distinct current levels were evident while the channel was open: a main current level (i_m) of 2.9 pA, and a sublevel (i_{s1}) of 1.7 pA. The fraction of time the channel spent at i_m and i_s, given that it was open, was 0.97 and 0.03 respectively. At +70 mV similar clusters of outward current bursts were also recorded with a main current level of 2.2 pA and a sublevel of 1.2 pA. In this case the fraction of time the channel spent at i_m and i_s was 0.93 and 0.07 respectively (day 37 neurone). *b*, The upper trace shows current bursts of glycine receptor channels recorded at −70 mV. The pipette and bath solutions were as described in Fig. 1 legend. Examination of individual events in such bursts at higher time resolution (lower traces) indicated events of up to 500 ms that were interrupted by either brief (<5 ms) closing gaps or steps down to current sublevels. Note that in the last event of the first burst (upper trace) the sublevel appears in isolation. The relatively long events appear to be a kinetic state of glycine receptor channels activated by high (≤50 μM) glycine concentrations and are rarely seen at low concentrations. For example, at 5 μM, the average individual burst duration is 30 ms made up of brief openings (<10 ms). The main current level evident within these bursts was 3.3 pA with two distinct sublevels of 2.4 pA and 1.8 pA. Individual current levels were estimated initially by a line fitted by computer through the average of the noise about that level. The mean current level was subsequently determined from the peaks of current level histograms. The bursts shown were selected because they showed pronounced multi-level behaviour. The fraction of time the channel spent at the different levels, given that it was open, was 0.80, 0.15 and 0.05 respectively (day 35 neurone). In this patch and most patches studies (Fig. 3*b*, *d*) only three (or fewer) distinct current levels were evident. Certain patches did display glycine-activated currents with amplitudes larger than the main current level (≥4 pA). Because they occur usually with lower frequency and we have not so far seen transitions within bursts up to these larger levels they have not been studied here.

channels are distributed in a mosaic-like fashion over the somal membrane surface. Both types of receptor channel can coexist within a small (5–20 μm^2) membrane area[12].

Glycine activates current steps of larger amplitude than GABA (Fig. 1), consistent with results of 'noise analysis' experiments[8,13]. Examination of individual current steps, however, shows that both GABA and glycine activate channels with several conductance states. At high agonist concentrations (>20 μM) following partial desensitization current pulses are grouped into bursts, which presumably reflect several successive openings of the same channel (Fig. 2*a* and refs 11, 14). At −90 mV, the most frequently observed GABA-activated current level (i_m) was 2.9 pA, but the current also stepped down from this level to a lower sublevel (i_s) of 1.7 pA or stepped up from resting level to this sublevel (Fig. 2*a*). Similarly at +70 mV when Cl^- currents were outward, the main current level within bursts was 2.2 pA with a sublevel of 1.2 pA. Glycine-activated currents displayed even more complex patterns. Within bursts of single-channel currents, the current could step down from the main level (i_m) of 3.3 pA to sublevels (i_{s2}, i_{s1}) of either 2.4 or 1.8 pA (Fig. 2*b*). Alternatively, the current could step up from the resting level to either of the two sublevels before jumping to the main level or back down to the resting level.

Multiple current levels were also observed when channels were activated by non-desensitizing concentrations of glycine or GABA where channel openings occurred in a random fashion. For example, Fig. 3*a*, *b* shows amplitude histograms of currents activated by 2 μM GABA and 10 μM glycine respectively. The histogram of GABA-activated currents was fitted by two well separated gaussian distributions with peaks of 1.4 pA and 2.2 pA respectively. The relative fraction of the total time the channel spent at these levels, given that the channel was open, was 0.05 and 0.95. The histogram of glycine-activated currents measured in similar conditions (Fig. 3*b*) was fitted by three well separated gaussian distributions with peaks of 1.7, 2.2 and 3.1 pA. The relative fraction of time spent at each level was 0.06, 0.12 and 0.82 respectively. Current–voltage (*i*–*V*) relationships measured in equal Cl^- concentrations (145 mM) on both membrane faces (Fig. 3*c*, *d*) indicate that the different current steps represent channel conductances of 45, 31 and 21 pS for the glycine receptor channel, and 30 and 19 pS for the GABA receptor channel. Bicuculline (10 μM) and strychnine (5 μM), which selectively block the GABA- and glycine-activated currents respectively, did not alter the multistate behaviour of glycine and GABA channels. These results indicate that both GABA and glycine activate channels which can adopt several

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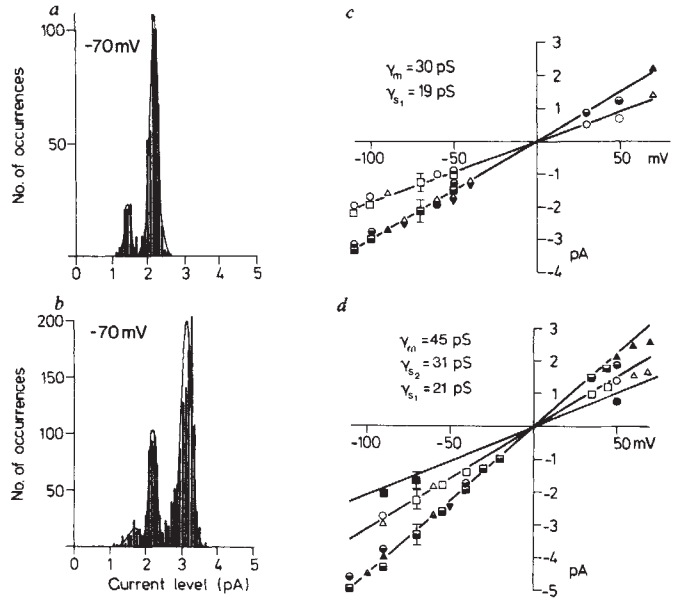

Fig. 3 The predominant conductance states displayed by GABA (*a*, *c*) and glycine (*b*, *d*) receptor channels measured with Cl^- concentration of 145 mM on both membrane faces. *a*, Histogram showing the number of occurrences of the different current levels activated by 2 μM GABA at −70 mV membrane potential. The fitted gaussian distributions had peaks of 1.4 and 2.2 pA. This patch was not sensitive to glycine. For this experiment and that described in *b*, the pipette and bath solutions were as defined in Fig. 1 legend. *b*, Histogram of the number of occurrences of the different current levels activated by 10 μM glycine at −70 mV. Three distinct peaks of 1.7, 2.2 and 3.3 pA were evident. This patch was not sensitive to GABA. Day 37 neurone. *c*, *i–V* relationships measured for GABA receptor channel currents. All recordings were made with equal (145 mM) Cl concentrations. Different symbols represent different cation concentrations: □, $Tris^+$ bath, K^+ pipette; ○, $Tris^+$ bath and pipette; ◇, ▽, Na^+ bath, $Tris^+$ pipette; △, Na^+ bath, K^+ pipette. The lines were fitted to the data points, with the restriction that they passed through 0 mV, and yielded conductances of 19 and 30 pS. The data points at −70 mV represent the mean ±s.d. of current levels measured from five different patches. The relative proportion of time the channel spent at its main level given that it was open varied between 1.0 and 0.7 on different patches measured in the same conditions. *d*, *i–V* relationships for glycine receptor channel currents. The symbols represent the same conditions as for *c*. The lines were fitted to the data points, with the restriction that they passed through 0 mV, and yielded conductances of 21, 31 and 45 pS. The data points at −70 mV represent the averages ± s.d. of four different patches. The fraction of time the channel spent at the different levels varied from patch to patch. For example, for 15 patches studied in similar conditions, 3 patches displayed only the main current level, 7 patches displayed i_m and i_{s2}, and 5 patches displayed all three conductance levels.

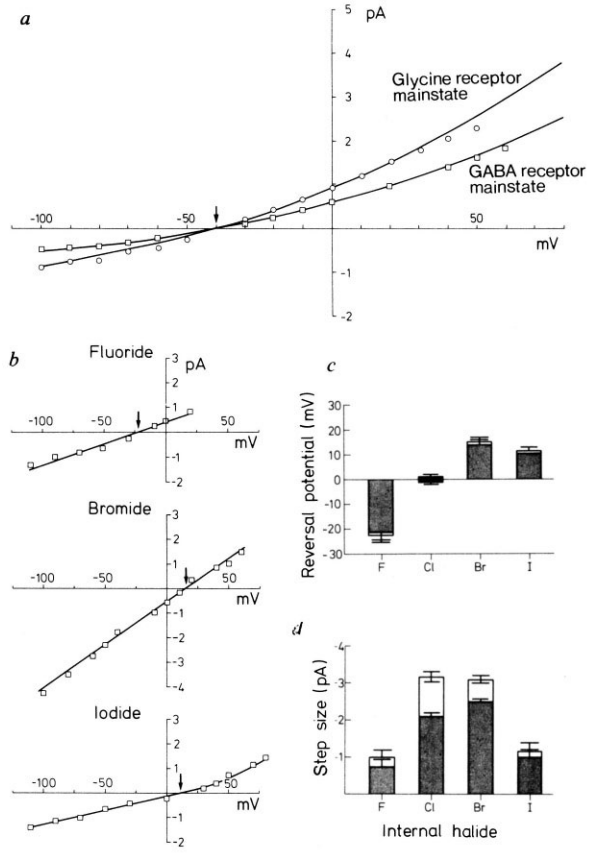

Fig. 4 The anion-selective properties of GABA and glycine receptor main conductance states. *a*, *i–V* behaviour of glycine- and GABA-activated main current levels measured with unequal Cl^- concentrations on the two membrane sides. The solution at the cytoplasmic membrane face (that is, in the pipette) contained (in mM): 25 CsCl, 55 Cs_2SO_4, 55 sucrose, 1 $CaCl_2$, 1 $MgCl_2$, 1 KCl, 11 Na-EGTA and 10 Na-HEPES buffer, *p*H 7.2. The bath solution was the same as described in Fig. 1 legend. The graphs are plots of the main current levels for GABA- and glycine-activated current steps measured in different patches. The curves fitted to the data points were derived from the constant-field equation, assuming single glycine and GABA receptor channel permeabilities of 11.0×10^{-14} and 7.73×10^{-14} $cm^3\,s^{-1}$ respectively (using Cl^- activities). This corresponds to the single channel conductances of 45 and 30 pS obtained for equal (145 mM) Cl concentrations (Fig. 3). Note the outward rectification. The slope conductances of GABA and glycine receptor channels measured at +50 mV were 23 and 36 pS respectively, while at −100 mV values of 5 and 12 pS were obtained. Although for simplicity only the main current level behaviour is described in *a*, multistate behaviour was also observed in these ionic conditions. For example, at +50 mV glycine receptor channels had conductance levels of 2.3 (i_m), 1.6 (i_{s2}) and 1.0 (i_{s1}) pA. GABA receptor channels had current levels of 1.6 (i_m) and 0.9 (i_{s1}) pA. *b*, Halide selectivity of glycine-activated currents. In these experiments 140 mM F^-, Br^-, or I^- (all Na salts) replaced all but 5 mM internal Cl^-. External Cl was kept constant at 145 mM. *i–V* relationships for glycine-activated currents are shown in the different halide conditions. The reversal potentials determined by interpolation from these graphs were −23 mV (F^-), +15 mV (Br^-) and +10 mV (I^-). *c*, Reversal potentials determined from *i–V* relationships such as described in *b*. The reversal potentials in such conditions gave a permeability sequence for both channels of $Br^- > I^- > Cl^- > F^-$ with permeability ratios calculated from the constant field equation of 1.8:1.5:1.0:0.4 for glycine receptor channels and 1.7:1.4:1.0:0.4 for GABA receptor channels. □, GABA receptor mainstate; ▨, glycine receptor mainstate. *d*, Main current levels of glycine- and GABA-activated channel currents at −70 mV. Values are averages from at least three estimates for each ionic condition and both channel classes. Single-channel conductance estimated from the slope conductance at −70 mV gave a sequence of channel conductances of $Cl^- > Br^- > F^- > I^-$ with conductance ratios of 1.0:0.80:0.41:0.31 for glycine receptor channels and 1.0:0.96:0.48:0.42 for GABA channels. i_{s2} of the glycine channel in these different halide conditions at −70 mV was 0.8 (F), 2.4 (Br) and 0.8 (I) pA, in good agreement with i_m of GABA receptor channels. i_{s1} could not be accurately measured for F^- and I^- currents.

conductance states. That the two receptor channels differ in their most frequently observed main conductance states of 30 pS and 45 pS, but share common conductance states of approximately 30 pS (γ_{s2} of the glycine receptor channel and γ_m of the GABA receptor channel) and 20 pS (γ_{s1} of glycine and GABA receptor channels), may indicate that they are identical.

Further evidence for a similar channel structure is indicated by the ion selectivity of the channels. First, glycine and GABA receptor channels are highly selective for anions. Complete replacement of Cl^- at both membrane faces by SO_4^{2-} or acetate abolished both current responses. On the other hand, *i–V* relationships of GABA and glycine receptor channels (Fig. 3*c*, *d*) were independent of the cations (Na^+, K^+ or $Tris^+$) present.

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When internal Cl^- concentration was reduced from 145 mM to 30 mM, the zero-current potential was shifted from 0 to −40 mV (Fig. 4*a*). In these conditions, the *i–V* relationships for both glycine and GABA receptor channels displayed outward rectification which could be described adequately by the constant-field equation for Cl^--selective channels.

Second, both channels show the same selectivity between different halides. Thus in substitution experiments in which Cl^- at the cytoplasmic face was replaced by F^-, Br^- or I^- while keeping the extracellular Cl^- concentration constant (Fig. 4*b–d*), for both GABA- and glycine-activated channels reversal potentials (Fig. 4*c*) determined from *i–V* relationships (shown for glycine receptor channels in Fig. 4*b*) indicated a halide permeability sequence of $Br^- > I^- > Cl^- > F^-$. This contrasts with a conductance sequence of $Cl^- > Br^- > F^- > I^-$ determined from slope conductance at −70 mV. The difference between the observed permeability and conductance sequences indicates that in both receptor channels, permeant anions interact with ion-binding sites in the channel.

We conclude that GABA and glycine receptor channels show the same ion selectivity and share similar conductance states. This may indicate that a single species of multistate Cl^- channel is coupled to GABA or glycine receptor subunits. This channel, when liganded with an agonist, adopts a main conductance state which is determined by the receptor subunit to which it is coupled. The concept that the same effector is linked to different membrane receptors has previously been demonstrated for receptors and cyclases[15–17]. It could also be true for receptors and ion channels[18].

We thank E. Neher and F. Sigworth for computer programs and comments on the manuscript, and S. Clapham and G. Kotte for typing the manuscript.

Received 5 April; accepted 12 August 1983.

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Development of electrical membrane properties in cultured avian neural crest

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In previous studies of the development of membrane excitability in vertebrate neurones[1,2], a calcium current has commonly been observed first, later replaced by a sodium current. We have now examined the development of membrane currents in explant cultures of mesencephalic neural crest cells from the quail embryo. Some of these cells constitute the precursors for the ciliary and trigeminal ganglia[3,4] and in certain conditions can be characterized morphologically as neurones after only a few hours in culture[5,6]. We report here that two membrane currents are present in neurones after 1 day in culture, a voltage- and time-dependent potassium current and a leakage current. On the second day in culture, voltage-dependent sodium and calcium currents can be detected. With time the sodium and calcium currents increase in magnitude and all four currents are present for at least 7 days in culture. This onset of electrical excitability differs from that described in other vertebrate neurones both *in vitro*[7] and *in vivo*[8,9], but resembles the sequence observed in neurones of the developing grasshopper[10].

Mesencephalic neural crests were excised from quail embryos at the 10–12-somite stage, by which time crest cells are migrating dorsolaterally from the encephalic vesicle. Neural crests were cultured as explants in a chemically defined medium[5,6]. Recordings were made from morphologically identifiable neurones using patch electrodes[11] (whole-cell recording[12,13]). We do not know whether these neurones are autonomic, sensory or a mixed population.

After 1 day in culture, the cells studied were small, elongated and flat, and were considered presumptive neurones on the basis of immunocytochemical labelling of tetanus toxin-binding sites and of neurofilament proteins[5,6]. The cells had resting membrane potentials of −41 ± 12 mV (mean ± s.d.; $n = 11$) in control medium[13]. When a pulse of depolarizing current was applied, the membrane voltage changed (Fig. 1*a*). At the onset of the pulse there was a peak depolarization that decayed to a steady voltage. A similar result was obtained when the cell was hyperpolarized to −80 mV before application of the depolarizing pulse, indicating that the failure to evoke an action potential was not simply the result of channel inactivation at the resting potential. This behaviour was also seen consistently on the second day in culture. On day 4, however, resting potentials were still near −40 mV but in some cells application of a depolarizing pulse from a holding potential of −80 mV elicited a brief action potential (Fig. 1*b*).

Voltage-clamp in combination with pharmacological agents was used to identify the membrane currents underlying the electrical events illustrated in Fig. 1. Figure 2*a* shows recordings from a cell cultured in control medium for 24 h. Application of depolarizing pulses to the cell activated a voltage- and time-dependent outward current (Fig. 2*a*) that could be blocked by adding a combination of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) to the medium (Fig. 2*b*), two agents known to block potassium channels. Further evidence that the outward current was carried by potassium ions was provided by reversal potential measurements of tail currents (data not shown): in 16 cells, the reversal potential of the outward current was −73 ± 4 mV, a value close to the potassium equilibrium potential calculated from the known extracellular potassium concentration (5.4 mM) and from the potassium concentration inside the patch electrode (100 mM). Examination of the current–voltage (*I–V*) relationship of a presumptive neurone after 24 h in culture, measured in control medium (Fig. 2*c*, circles) and in medium containing 4-AP and TEA (Fig. 2*c*, squares), shows that the threshold for the potassium current is near −50 mV. In the presence of the blocking agents, there remained a leakage current having an average reversal potential of −12 ± 18 mV ($n = 25$). In the cell illustrated in Fig. 2*c*, the reversal potential for the leakage current was −12 mV. Adding tetrodotoxin (TTX) or cobalt did not affect these membrane properties. We therefore conclude that after 24 h in culture voltage- and time-dependent inward currents are not yet present. The behaviour of the cell observed in Fig. 1*a* can, however, be explained on the basis of the presence of the voltage- and time-dependent potassium current and of the leakage current (C.R.B. *et al.*, in

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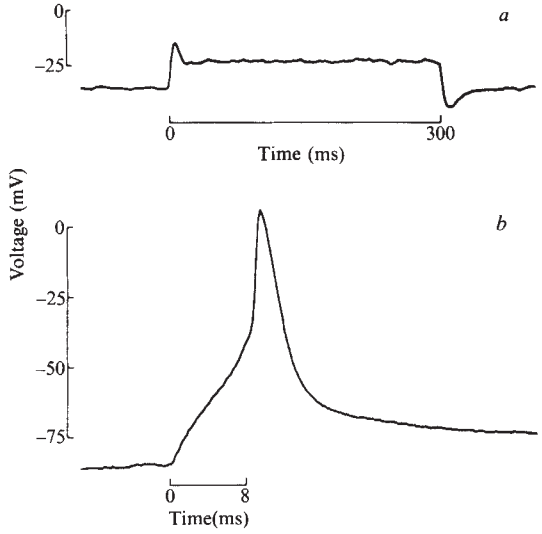

Fig. 1 Current clamp recordings at two different stages of development. *a*, Cell 24 h in culture; recording was done in control medium, at a temperature of 32 °C. Control medium was minimal essential medium with added glutamine (2 mM), glucose (0.6%). penicillin (30 μg ml^{-1}), streptomycin (50 U ml^{-1}), bovine serum albumin (20 μg ml^{-1}) and $CaCl_2$ (2 mM). A pulse of depolarizing current (80 pA) was applied for 300 ms. Similar recordings were obtained in 10 other cells. *b*, Cell 4 days in culture; recording was done in control medium. The cell was hyperpolarized to −80 mV by injecting a constant current and a depolarizing pulse of current (80 pA) was applied for 8 ms.

Methods: Explants were grown on collagen-coated plastic coverslips[13]. Recording was made by the whole-cell recording technique[12,13], using patch electrodes[11] with ~1-μm opening. The membrane–pipette resistance before breaking the patch was >5 GΩ. Electrodes were filled with 100 mM KCl, 5 mM NaCl and 45 mM EGTA (EGTA was first prepared as a 0.5 M stock solution (*p*H adjusted to 7.4 with KOH); with EGTA addition the *p*H of the intra-pipette solution was 7.4. The pipette resistance was <20 MΩ). These electrodes gave similar results to those observed with fine-tip electrodes in test experiments using neurones cultured for 7 days; these neurones were much larger than younger neurones. Current injection and voltage recording were done with the same electrode using a chopping technique[13,23,24]. The chopping frequency was 27.7 kHz. Voltage and current records were digitized (12-bit resolution) and stored on floppy disks using a 16-bit microcomputer (8086; Lomas Data Products). Voltage scale represents the voltage with reference to the extracellular fluid, taken as 0 mV.

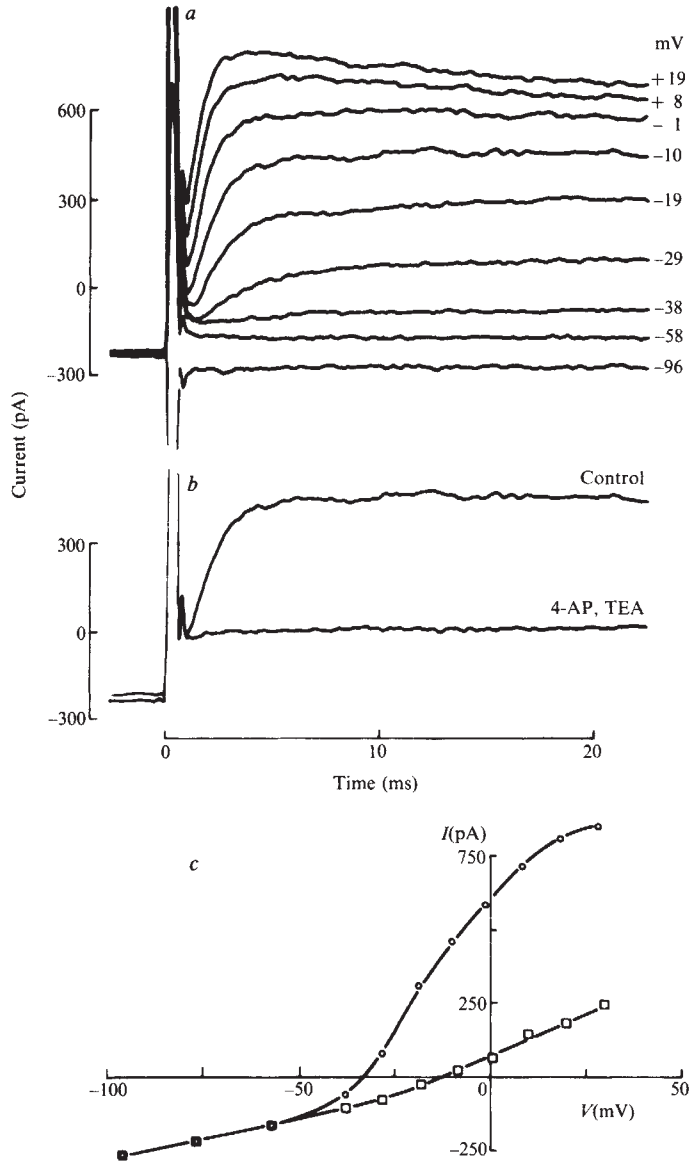

Fig. 2 Potassium current in a cell 24 h in culture. All recordings are from the same cell. *a*, Current traces recorded during steps in voltage clamp from −80 mV to the values indicated on the right. Recording was done in control solution. *b*, Comparison of current traces recorded in control medium and in a medium containing 2 mM 4-AP and 20 mM TEA; the block was reversible. The steps are from −80 to −10 mV. The large transient currents in *a* and *b* are the capacitative currents required to bring the membrane potential to the voltage of the step. *c*, Current–voltage relationship in control medium (○) and in medium containing 4-AP and TEA (□). The current was measured at its maximum value. Lines were drawn by eye.

preparation). Moreover, if the membrane resting potential is essentially dependent on these two currents, its low value of −40 mV can be simply explained (it must be more depolarized than −50 mV, the threshold for the potassium current).

After 36 h in culture an inward current could be detected, and by 48 h it was large enough to be studied. This inward current has fast activation kinetics and is followed by a delayed outward current (Fig. 3*a*), similar to the potassium current observed in Fig. 2*a*. The inward current is a sodium current as it can be suppressed by removal of sodium (data not shown) and by addition of TTX (see below). The *I–V* relationship of the cell illustrated in Fig. 3*a* is shown in Fig. 3*b*. When peak inward currents and delayed outward currents are plotted together with the *I–V* relationship of the same cell recorded in the presence of 4-AP, TEA and TTX, it can be seen that the inward sodium current is activated near −40 mV, a value similar to the activation potential of the delayed potassium current in this cell. Clearly, after 48 h in culture the potassium current is still present (it was found in all cells examined; $n = 11$); at that time 12 out of 13 cells studied showed a sodium current.

By contrast, a calcium current could be detected in sister cultures after 48 h in only 9 out of 13 cells. Thus in the cell illustrated in Fig. 3, for example, substitution of cobalt for calcium did not affect the current–voltage relationship, indicating that there was no detectable calcium current in this cell. On the other hand, Fig. 4*a* shows calcium currents at several voltages recorded in a cell after 48 h in culture; sodium and potassium currents were blocked by TTX, 4-AP and TEA. Figure 4*b* shows the *I–V* relationship of the same cell recorded in the presence of calcium or cobalt. When TTX was present alone, no action potential could be triggered; long-duration calcium action potentials could be triggered only when the potassium current was blocked. On the fourth day in culture, long action potentials could be evoked in all cells. After 4 days in culture the relative magnitude of the three voltage-dependent currents could be estimated from the maximal value of each current; for I_K, this was 680 ± 245 pA (mean ± s.d.); for I_{Na},

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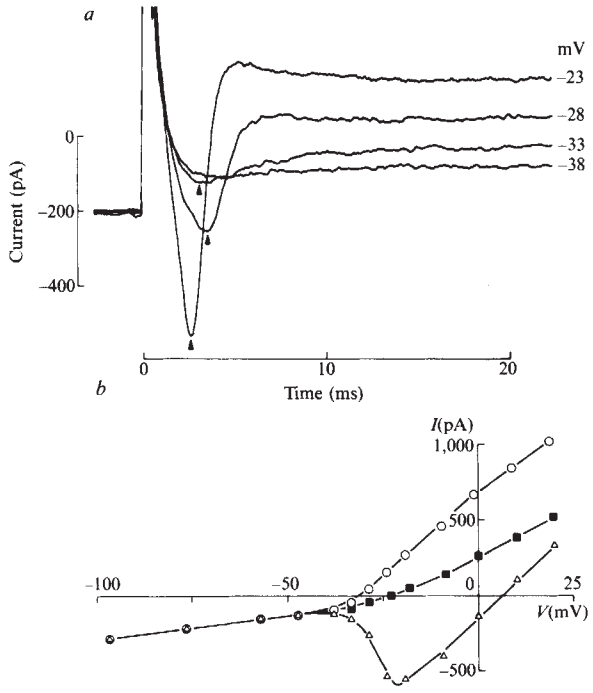

Fig. 3 Potassium and sodium currents in a cell cultured for 48 h. *a*, Voltage-clamp recording in control medium. Series of current recordings during steps in voltage clamp from −80 mV to the voltage values indicated on the right. For depolarizations exceeding −40 mV there is an early inward current (arrow). *b*, Current–voltage relationship in the same cell as in *a*; values were measured at the peak of the inward current (△) and at the maximum of the outward current (○). ■, Currents measured when recording in a medium containing TTX (5 μM), 4-AP (2 mM) and TEA (20 mM) to block the sodium and potassium currents; substituting calcium with cobalt did not alter the current–voltage relationship in this cell, indicating that there was no calcium current. The difference between the triangles and the squares represents the inward sodium current; the difference between the circles and the squares represents the outward potassium current (compare with Fig. 2*c*).

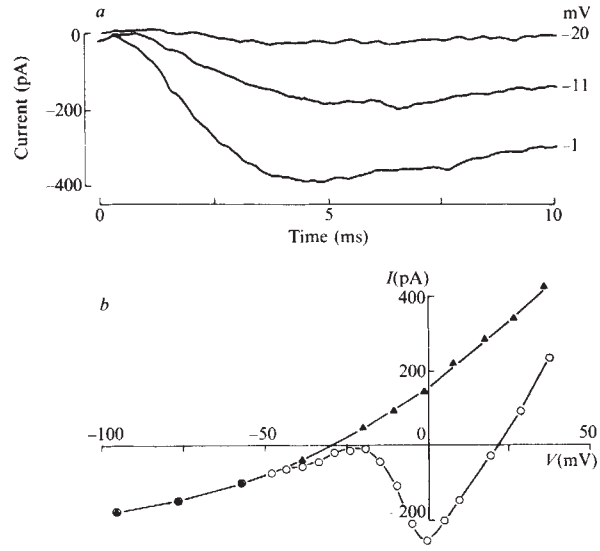

Fig. 4 Calcium current in a cell cultured for 48 h. The medium contained TTX (5 μM), 4-AP (2 mM) and TEA (20 mM) to block the sodium and potassium currents. *a*, Difference in currents at a given voltage (values on the right) between recordings with 3 mM calcium or 3 mM cobalt. The three traces indicate that cobalt blocks an inward current. *b*, Current–voltage relationship in the presence of calcium (○) or 3 mM $CoCl_2$ (▲). The block by cobalt was reversible. This inward current, identified as a calcium current, activates near −40 mV and there seems to be a second threshold near −20 mV. This behaviour was consistently observed in six other cells.

670 ± 90 pA; and for I_{Ca}, 170 ± 60 pA. Note that the assessment of currents in the present study is principally pharmacological; a change in sensititvity to a blocking agent during development cannot be excluded. The observed persistence of a calcium current is consistent with our previous findings in chick ciliary ganglion neurones from 11-day-old embryos[14]. These neurones, which derive from the mesencephalic neural crest, exhibited a calcium current for several weeks in culture.

The present results show that mesencephalic neural crest cells that have been characterized as neurones on morphological criteria[5,6] are not electrically excitable after 24 h in culture but can fire brief action potentials after 4–5 days. Comparison between the morphological and electrophysiological studies suggests that the tetanus toxin-binding sites and the neurofilament proteins appear before sodium and calcium currents can be detected. The first currents to appear are a voltage- and time dependent potassium current and a leakage current; we do not yet know whether one of these currents precedes the other during development. The next current to appear is probably a sodium current, closely followed by a calcium current. The voltage of activation for the calcium current is near −40 mV, which is the average membrane resting potential of the neurones for at least the first 4 days in culture. If there is little inactivation of the calcium current[15,16], at resting potential a significant influx of calcium through the voltage-dependent channel may take place by the second day in culture and affect the intracellular calcium concentration. A change in intracellular calcium may influence the development of the neurones[8,17,18], promote the activation of calcium-dependent currents[15,19,20] or modulate the metabolism of the neurones[21].

The sequence of development of membrane currents in cultured quail mesencephalic neural crest cells resembles somewhat that of neurones of the developing grasshopper[10]; however, it differs from the sequence described in amphibian neurones[7–9] where an initial calcium current is followed by a sodium current. Earlier experiments in another amphibian preparation, however, revealed outward rectification at stages when no regenerative activity could be detected[22]. The available evidence suggests that outward potassium currents precede the appearance of inward calcium and sodium currents in the amphibian[1,2] as well as in the quail.

We thank Professors F. Baumann, N. Le Dourain and Y. Dunant for encouragement and support; Mrs F. Steimer for technical assistance; and Drs W. Brownell, G. Jones, N. Spitzer and M. Weber for comments on the manuscript. This work was supported by grants from the Swiss NSF (3.210.0.0.82 to C.R.B. and D.B.; 3.230.0.82 to A.C.K.).

Received 24 June; accepted 19 August 1983.

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Stimulation of DNA synthesis by cathepsin D digests of fibronectin

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A number of studies over the past decade (reviewed in refs 1–3) have suggested an intimate link between fibronectin, proteolysis and the control of cell proliferation. So far, however, no unequivocal connection has been made. Recent evidence[4,5] that gelatin-binding fragments of fibronectin enhance morphological cell transformation led to the postulate that the fibronectin molecule harbours latent properties released only on proteolysis. These findings led us to investigate the role of fibronectin fragmentation in the regulation of cell growth *in vitro*, and we report here that whilst fibronectin is without effect, its cathepsin D digests possess the ability to initiate DNA synthesis in serum-deprived quiescent cultures of normal hamster fibroblasts.

Fibronectin (for reviews, see refs 6–8) was isolated from bovine plasma by the combined gelatin and heparin affinity chromatography protocol described by Yamada[9]. The product, as monitored by SDS–polyacrylamide gel electrophoresis in reducing conditions, was either a closely spaced doublet or a single band of molecular weight 220,000 (Fig. 1). Digestion with cathepsin D was carried out at 37 °C, *p*H 3.5, with an enzyme/substrate ratio of 1 : 125 (w/w). Electrophoretic analysis showed fragmentation with time (Fig. 1): after initial cleavage to several discrete bands, extended incubation resulted in further degradation to a large number of polypeptides. When fibronectin was treated with cathepsin D pre-inhibited with pepstatin there was no digestion (Fig. 1).

After extensive dialysis against phosphate-buffered saline (PBS), equal aliquots of a series of time-course digests were tested for their effect on the initiation of DNA synthesis in serum-deprived cultures of normal CH23 hamster fibroblasts. In agreement with previous reports[10,11], intact fibronectin induced a similar level of ^{3}H-thymidine incorporation to that of a PBS control (Fig. 2). The cathepsin D-generated digests, however, caused significant stimulation of DNA synthesis 24 h after their addition (Fig. 2). The activity of the digests depended on dose (Fig. 3) and the time of their cleavage from fibronectin (Fig. 2), peaking after approximately 2 h digestion and decreasing thereafter. The activity was not due to an artefactual effect of the digests on the rate of ^{3}H-thymidine uptake by cells because the transport rate in stimulated cells was indistinguishable from that of quiescent cells (results not shown). Furthermore, amethopterin, a potent inhibitor of thymidine monophosphate synthesis[12], was included in the incubation medium to eliminate any spurious effects due to interference of endogenously synthesized thymidine. The digest activity was not due to any residual proteolytic activity attributable to free cathepsin D or to cathepsin D–pepstatin complexes as an enzyme-inhibitor blank in the presence or absence of fibronectin had only basal activity (Fig. 2).

Cathepsin D fragments of fibronectin were able to promote the initiation of DNA synthesis at a minimum concentration of 160 ng ml^{-1}, although substantial stimulation was only observed at 1–2 μg ml^{-1}. This is approximately the same level De Petro *et al.*[4] have found to enhance morphological cell transformation. In our studies, however, a similar alteration of cellular shape was never apparent in digest-treated CH23 cells. Uncleaved fibronectin was mitogenically inactive up to a concentration of 50 μg ml^{-1}.

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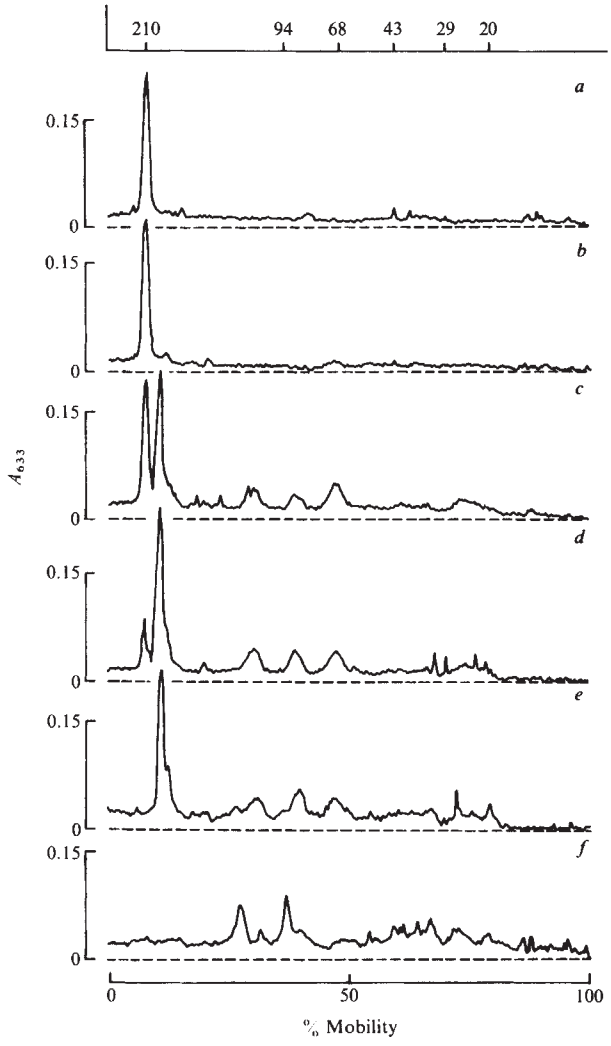

Fig. 1 SDS–polyacrylamide gel electrophoresis of cathepsin D-generated digests of fibronectin. Fibronectin was isolated from bovine plasma using consecutive gelatin and heparin affinity chromatography as described by Yamada[9]. Before digestion with cathepsin D (Sigma), fibronectin was dialysed into 5 mM Tris-HCl *p*H 3.5, 10 mM NaCl, 0.2 mM EDTA. Cathepsin D was then mixed with fibronectin at an enzyme/substrate ratio of 1 : 125 (w/w) and digestion was performed at 37 °C. Samples were taken at various intervals and digestion stopped by addition of a 10-fold molar excess of pepstatin (Sigma). Each digest was then dialysed into phosphate-buffered saline (PBS: 140 mM NaCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl) and stored at −20 °C. Samples were prepared for electrophoresis by dilution with sample buffer (0.0625 M Tris-HCl *p*H 6.8, 5 mM SDS, 100 mM dithiothreitol, 10% glycerol) and heating at 100 °C for 3 min. Electrophoresis was carried out in slab gels by the method of Laemmli[31] using a 3% stacking gel and a combined 8%/13% separating gel. Following electrophoresis, the gel was stained for 1 h at 37 °C with 0.115% Coomassie blue R (Sigma) in 20% ethanol, 8% acetic acid, destained in several changes of 20% ethanol, 8% acetic acid and bands visualized by laser scanning at 633 nm. *a*, Fibronectin; *b–f*, fibronectin digested for 0, 1, 2, 4 and 23 h respectively with cathepsin D. Mobility (expressed as % of the distance moved by the dye front) and molecular weight (×10^{-3}) of a series of molecular weight markers are shown at the top of the figure.

Co-incubation of the stimulatory cathepsin D digest with either gelatin, heparin, or fibronectin resulted in a substantial degree of inhibition. Fibronectin produced the most potent inhibition, the activity of 1 μg ml^{-1} of fibronectin fragments being inhibited by 50% by 0.25 μg ml^{-1} intact fibronectin. Although gelatin and heparin caused equal inhibition of the same concentration of digest, they were not as effective as fibronectin (50% inhibition at 2.5 μg ml^{-1}). From these results

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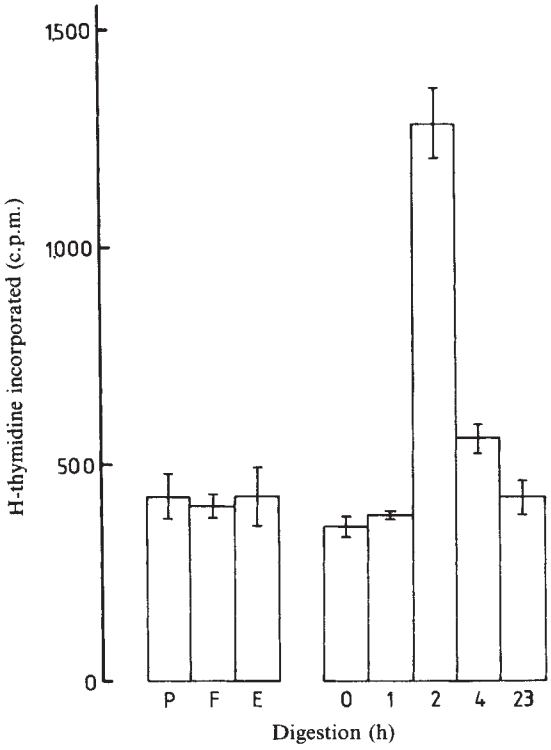

Fig. 2 Stimulation of DNA synthesis in quiescent CH23 fibroblasts by cathepsin D-generated digests of fibronectin. CH23 cells[32] were seeded into 35-mm plastic Petri dishes (Nunc) and cultured at 37 °C under an atmosphere of 5% CO_2/95% air in Dulbecco's minimal essential medium (Gibco) supplemented with 10% newborn calf serum (Gibco), 2 mM glutamine (Gibco), 0.25% $NaHCO_3$, 10 $\mu g\ ml^{-1}$ gentamycin sulphate (Sigma) and 0.2 $\mu g\ ml^{-1}$ streptomycin sulphate (Glaxo). Just before attainment of confluency, the growth medium was aspirated and replaced with 1.5 ml serum-free growth medium (containing 4 mM glutamine). Cells were allowed to quiesce for 21–22 h. Equal aliquots of each cathepsin D digest were then added and the medium volume made up to 2 ml with PBS. After a further 24-h incubation the rate of DNA synthesis was measured by a 1-h incorporation of 1.25 $\mu Ci\ ml^{-1}$ ^{3}H-thymidine (44 Ci $mmol^{-1}$, Amersham) in the presence of 10 μM amethopterin in dimethyl formamide (DMF) to inhibit endogenous synthesis of thymidine monophosphate[12]. The final level of DMF (0.25%) had no effect on either the viability of cell cultures as assessed by Trypan blue dye exclusion (95% minimum) or the rate of DNA synthesis. Incorporation of label was stopped by rapid washing with 1.5-ml aliquots of cold PBS and acid-soluble thymidine released by treatment with 1.5 ml 5% trichloroacetic acid (TCA) for 2 h at 4 °C. Residual TCA was removed by washing with 1.5-ml aliquots of 95% ethanol and acid-insoluble material solubilized with 2 ml 0.1 M NaOH for 1 h at room temperature. 0.5 ml samples were mixed with 3 ml Ready-Solv EP scintillant (Beckman) and counted for ^{3}H. The level of incorporation induced by 10% newborn calf serum was 4,270 c.p.m. in this experiment. Bar lines in all DNA synthesis experiments specify duplicate determinations from a representative experiment. P, PBS control; F, fibronectin; E, cathepsin D–pepstatin control.

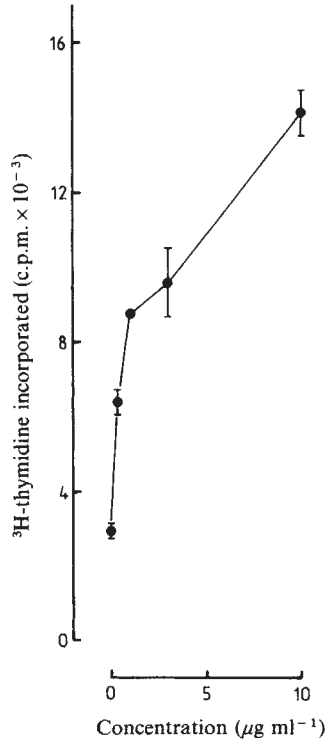

Fig. 3 Dose-dependent stimulation of DNA synthesis by cathepsin D digest. Varying volumes of the 4-h cathepsin D digest described in Fig. 1 were added to quiescent cultures of CH23 cells and the rate of DNA synthesis determined 24 h later as described in Fig. 2 legend. The level of incorporation of 10% newborn calf serum was 124,000 c.p.m. in this experiment.

it is therefore uncertain which of the recognized domains constituting the fibronectin molecule are responsible for the observed mitogenic activity. It remains possible that one or more sites or an, as yet, uncharacterized domain is involved.

Fragmented fibronectin has been isolated from several sources including normal human plasma[5,13–15]. Indeed, its presence in body fluids appears to be a marker for diseases characterized by elevated proteolytic activity, including cancer[16,17]. Thus our results and those of others[5,14,18–22] support a physiological role for fibronectin fragmentation and the proposal by Vaheri *et al.*[17] that cleavage of fibronectin can generate 'activation peptides' with regulatory function. They suggest that this is merely one manifestation of a number of fibronectin properties depending on prior macromolecular interaction. Moreover, the results support the conception of the extracellular matrix as a dynamic, rather than static, entity. The capacity of the extracellular matrix to respond to environmental change is likely since its synthesis and degradation are apparently under complex hormonal and proteolytic control[23–26]. Whether our results are functionally significant *in vivo* remains to be determined, but since cell-surface protein cleavage appears to be the primary action of several mitogenic proteases[27,28], the elevated levels of humoral and cellular proteases in the vicinity of tissue damage[29] coupled with the recognized involvement of fibronectin in wound healing[30] suggests that fibronectin fragmentation could be an ideal trigger for the initiation of cell multiplication.

We thank Professor Mike Grant and Dr Ken Yamada for comments on the manuscript and Dr Shirley Ayad for acid-soluble collagen type I. This work was supported by the Cancer Research Campaign. M.J.H. is supported by the SERC.

Received 5 July; accepted 22 August 1983.

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The heavy chain of human B-cell alloantigen HLA-DS has a variable N-terminal region and a constant immunoglobulin-like region

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The HLA-D region of the major histocompatibility complex (MHC) of man encodes polymorphic glycoproteins found predominantly on the cell surfaces of B cells and macrophages. These proteins mediate interactions, required for the induction of immune responses, among cells of the immune system and consequently are referred to as Ia (immune-response associated). Two families of Ia molecules, DR and DS (also known as DC), have been defined, the former analogous to the I–E (ref. 1) and the latter to the I–A molecules of the murine MHC[2–4]. Both DR and DS molecules consist of two non-covalently associated polypeptide chains with molecular weights of 33,000 and 28,000, designated α and β, respectively. The polymorphism of DR molecules is due to structural variation in the small subunit, DRβ, with the large subunit, DRα, being constant in structure[5–7]. In contrast, both subunits DSα and DSβ are structurally variable when DS allotypes are compared[3]. We have now isolated a cDNA clone from a DR7 cell line that contains the entire coding sequence for the DSα subunit and have compared its predicted amino acid sequence with that previously deduced from a DSα cDNA clone isolated from a DR4,w6 cell line[8]. This comparison reveals that 10 of 11 amino acid differences are located within the α1 (N-terminal) domain and that the α2 or immunoglobulin-like domains are identical.

Poly(A)-containing RNA was isolated from a consanguineous homozygous DR7 cell line (LG-10) and cDNA was synthesized by reverse transcription using an oligo(dT) primer followed by second-strand synthesis using DNA polymerase. A cloned cDNA library was constructed by insertion of the cDNA into the *Pst*I site of pBR322 using the poly(dG)·poly(dC) homopolymeric extension method[9]. Colonies containing cDNA were screened with a nick-translated cDNA probe, pDCH1 (provided by C. Auffray), which encodes the entire DS(DC) α subunit except for the leader sequence and 17 amino acids of the N-terminus[8]. From an initial screening of 10,000 colonies, 4 strongly hybridizing clones were detected, all of which had similar restriction patterns (data not shown); the one with the largest insert, pDSα-12, was subjected to DNA sequence analysis (Fig. 1). The cDNA insert of pDSα-12 consists of 921 nucleotides. Starting with the methionine initiation codon ATG at nucleotide positions 20–22, there is an open reading frame of 762 nucleotides encoding 254 amino acids and terminating with the TGA codon at nucleotide positions 782–784. This is followed by a 3′ untranslated region of 123 nucleotides and a poly(A) tail of 14 nucleotides. Nineteen bases upstream of the poly(A) tail is the canonical polyadenylation signal AATAAA. The first 23 codons, amino acids −23 to −1, encode the hydrophobic leader sequence. The amino acids predicted from the next 25 codons are in nearly perfect agreement with the previously determined N-terminal protein sequence of a DS7 α subunit[3]. By convention we have given the DS allotype the same numerical designation as the DR allotype of the cell line from which it was isolated. Accordingly, DS molecules isolated from a DR7 cell line are designated DS7. The two discrepancies are probably due to protein sequencing errors although the existence of a second DS7 α-like subunit cannot be ruled out. Thus, pDSα-12 contains the entire coding sequence for the DS7 α subunit.

As has been shown previously[8], the DS(DC) α subunit displays strong amino acid and DNA sequence homology to the DRα subunit (Fig. 2). However, despite the overall high degree of homology between DRα and DSα (54%) there are several regions of very low homology, notably the first 51 amino acids of the coding region, (including the leader sequence) where only 12 of 51 (24%) of the residues are identical, and amino acid positions 47–59, where only 4 of 13 (31%) of the residues are identical. In addition, the 3′ untranslated regions show no significant homology above random (30%).

When the nucleotide sequence of the pDSα-12 cDNA insert, which codes for the DS7α subunit, is compared with that of the

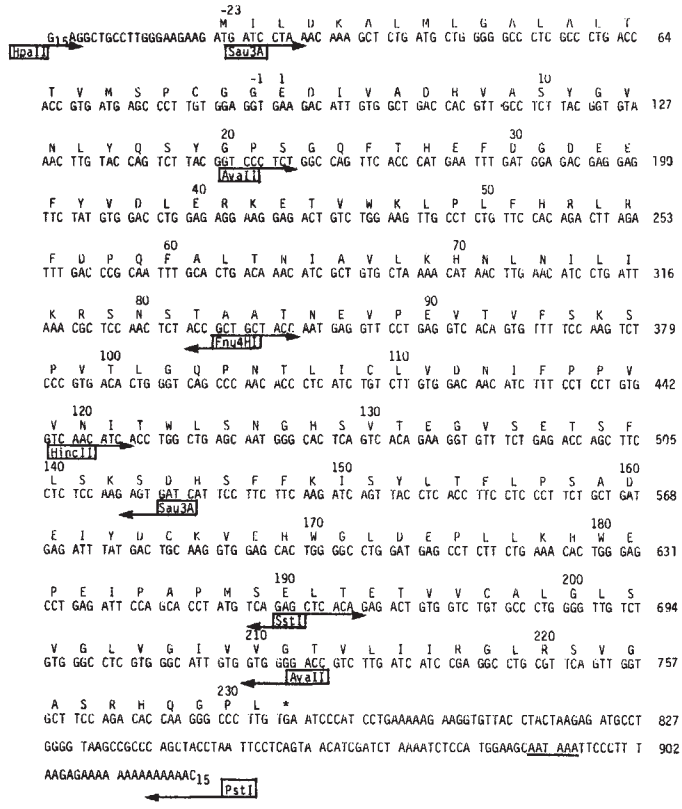

Fig. 1 cDNA and predicted amino acid sequence of the DSα subunit clone, pDSα-12. The nucleotide sequence of the coding strand and the predicted protein sequence (single letter amino acid code) are shown. The nucleotide sequence immediately follows the 15 guanine residues used to insert the cDNA into the *Pst*I site of pBR322. The 23-amino acid signal sequence is numbered −23 to −1 and the termination codon at nucleotide positions 782–784 is indicated by an asterisk. The canonical polyadenylation signal AATAAA located in the 3′ untranslated region is underlined. The restriction enzyme sites that were 5′ end labelled and used for DNA sequencing are shown. The nucleotide sequence of an extensive series of overlapping fragments was determined by the procedure of Maxam and Gilbert[18].

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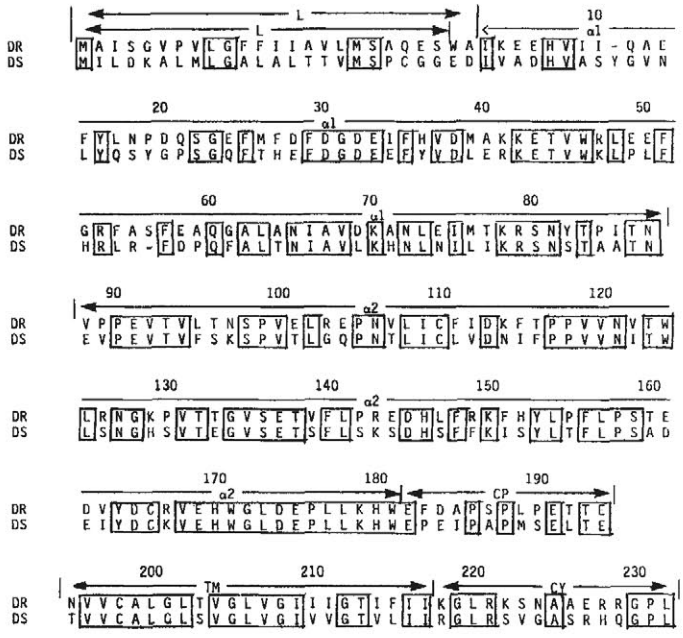

Fig. 2 Comparison of the protein sequences of DR and DS α subunits predicted from cDNA clones. The DRα protein sequence was predicted from the nucleotide sequence of a DRα cDNA clone previously isolated[19], and the protein sequence of the DSα subunit was predicted from the nucleotide sequence of the pDSα-12 cDNA clone described here. The DRα and DSα subunits may be organized into a leader (L) region, two extracellular domains (α1 and α2), a connecting peptide (CP), a transmembrane (TM) region and an intracytoplasmic (CY) region based on studies by Korman *et al.*[10], Auffray *et al.*[8] and Lee *et al.*[11]. Homologous regions of DR and DS α subunits are boxed. The homology between these two molecules is 21.7% in the leader sequence, 48.2% in the α1 domain 65.2% in the α_2 domain and 72.5% in the combined CP, TM and CY regions. The numbering system is based on the DS sequence.

pDCH1 cDNA insert, which codes for a nearly complete DS4, 6α subunit, 11 nucleotide substitutions as well as deletion of a codon at nucleotide positions 254–256 are observed (Fig. 3). A comparison of the predicted protein sequences for the DSα and DS4, 6α subunits (Fig. 4) reveals 11 amino acid sequence differences, 10 of which are located within the N-terminal portion of the molecule which by analogy to the DRα subunit has been designated the α1 domain[10,11]. The α2 domain, which represents an immunoglobulin-like region conserved among class I and class II histocompatibility antigens[12–15], is identical for both the DS4, 6 and DS7 α subunits. The 11th amino acid sequence difference is found in a region of the molecule referred to as the connecting peptide and bridges the α2 domain and the transmembrane segment. It is intriguing that 4 of the amino acid differences are clustered at positions 47–56, one of several regions, as pointed out above, where DR and DS α subunits differ substantially. Thus, there is even a suggestion of a hypervariable region. Indeed, recent studies by M. Davis *et al.* (personal communication) reveal that the murine Aα subunits (homologues of DS) show a high degree of variability at these same positions. Note also that of nine nucleotide differences within the α1 domain (the deletion of the codon at positions 254–256 is considered as a single substitution event), seven result in amino acid differences. This high percentage of nonsilent substitutions suggests that there are fewer structural and functional constraints on the α1 domain than for other proteins. We believe that pDSα-12 and pDCH1 represent products of alleles at the same locus because 126 of 127 nucleotides at the 3′ untranslated (UT) regions are identical. Although, at this time we cannot entirely rule out the possibility that these clones are products of different loci that have recently diverged[16,17],

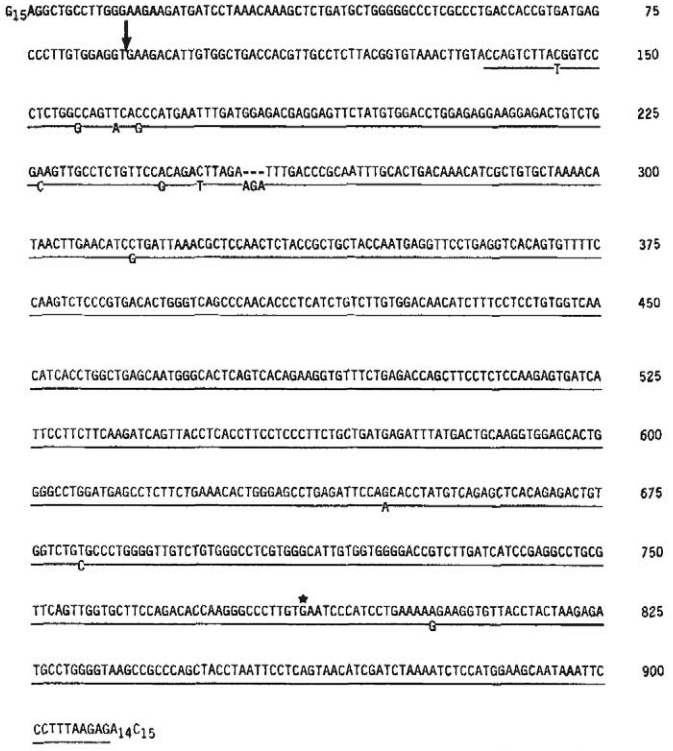

Fig. 3 Comparison of the DNA sequences of DS7 and DS4, 6 α subunits. The upper line represents the DS7α DNA sequence and the lower line the DS4, 6α DNA sequence (from ref. 8). The sequences are identical unless otherwise indicated. Positions 254–256 represent a deletion in DS7α with respect to DS4, 6α. The first N-terminal amino acid is indicated by an arrow and the termination codon by an asterisk.

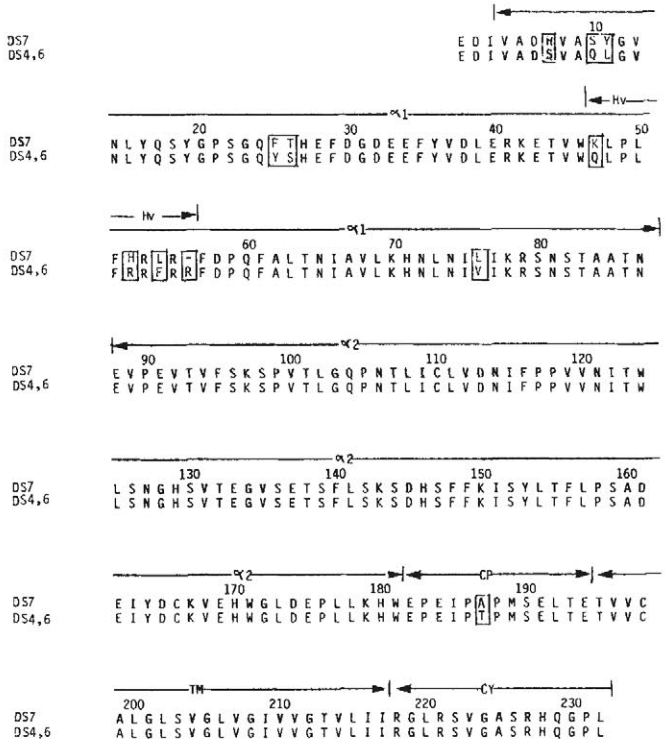

Fig. 4 Comparison of protein sequences of DS4, 6 and DS7 subunits. The predicted protein sequence of the DS7α subunit (Fig. 1) is compared with the predicted protein sequence of the DS4, 6α subunit taken from ref. 8. As the DS4, 6α cDNA clone did not contain the first 17 amino acids, residues 1–17 are based on protein sequence analysis[4]. Domain organization is as in Fig. 2. There are 11 amino acid differences between these two molecules, 10 in the α1 domain, the 11th in the connecting peptide region.

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it is difficult to see how they could have accumulated so many differences in the $\alpha 1$ domain while maintaining a relatively constant 3′ UT region. Additional studies should resolve this issue.

Thus, the DSα subunit consists of an N-terminal domain with a high degree of variability and a highly conserved immunoglobulin-like domain. The constancy of the $\alpha 2$ domain may reflect a functional requirement such as the ability to interact with the immunoglobulin-like domain of the DSβ subunit, interactions analogous to those that occur between immunoglobulin heavy and light chains. The role of variability in the $\alpha 1$ domain, however, remains to be determined.

This work was supported by a NIH grant.

Received 7 March; accepted 18 August 1983.

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Cytotoxic T lymphocyte recognition of transfected cells expressing a cloned retroviral gene

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The lysis of murine sarcoma virus–murine leukaemia virus (MSV–MuLV)-induced tumour cells by cytotoxic T lymphocytes (CTL) appears to require that an antigen specified by MSV–MuLV, or induced by the infection, be presented in association with class I major histocompatibility complex antigens[1,2]. The viral proteins of the tumorigenic MuLV seem to be a part of the antigens recognized by these dually restricted anti-MuLV CTL, but the precise nature of the putative viral antigen(s) recognized by CTL is unknown. Studies using recombinant viruses have suggested that a product of the viral envelope gene (*env* gene), perhaps the glycoprotein gp70, is the viral antigen recognized by CTL[3]. Attempts to use purified gp70 or anti-gp70 antibodies to block CTL recognition of retrovirus-induced tumour cells, however, have yielded contradictory results[4–6]. To examine more closely the role of gp70 in the CTL response to MuLV infections, we have constructed murine cell lines which express only the *env* gene of the Moloney murine leukaemia virus (M-MuLV). We show here that BALB/c-3T3 cells expressing the M-MuLV envelope gene products on their cell surface are susceptible to lysis by M-MuLV-specific CTL.

A biologically active molecular clone of M-MuLV derived from an integrated proviral genome[7] (given by Dr R. Mulligan) was used to construct an expression vector (Mo-env) containing the viral promoter and enhancing sequences, the long terminal repeats (LTR), viral polyadenylation signals, splice donor and acceptor sites, and only those translated sequences that encode the *env* gene (Fig. 1). Using a modification of the calcium phosphate transfection procedure[8], this DNA was introduced into BALB/c-3T3 fibroblast cells. To allow selection of those cells which were able to take up and integrate the transfected DNA, cells were co-transfected with the gene encoding the bacterial phosphotransferase APH (3′) I[9], which had been linked to the LTR of the Moloney Murine sarcoma virus (M-MSV), and conferred resistance to the antibiotic G418 (geneticin sulphate; Gibco). Transfection of a wide variety of mammalian cell lines with this MSV-neo recombinant yields stable transformants that are resistant to G418 at a frequency of 1 transformant per 10^4 transfected cells (D.V.F., unpublished results). Following co-transfection with Mo-env and MSV-neo, cells were grown in the presence of 0.5 mg geneticin sulphate per ml of medium. Approximately 10 days after transfection, discrete colonies of G418-resistant cells were visualized and isolated using cloning cylinders. These clones were expanded in tissue culture.

Table 1 Susceptibility of Mo-env-transfected BALB/c-3T3 cells to lysis by M-MuLV-specific CTL

Target cell	% Specific ^{51}Cr-release 20:1	40:1	80:1
M-MuLV-infected BALB/c-3T3	75.1	76.2	78.7
Uninfected BALB/c-3T3	1.8	2.6	7.7
Clone P4	1.0	3.0	8.4
Clone N4	9.7	14.2	34.8
Clone E4	12.3	18.9	38.4
Clone E5	29.4	39.5	56.4
LSTRA	75.5	73.5	70.5
MBL2	4.2	10.6	16.3

Spleen cells were taken from BALB/c mice which had developed spontaneously regressing sarcomas following intramuscular inoculation of 0.1 ml of a tumour extract containing the M-MSV–M-MuLV complex. These cells were cultured in 24-well Linbro plates at a density of 6×10^6 responder spleen cells per well together with 3×10^5 irradiated (7,000 rad) LSTRA tumour cells in supplemented RPMI-1640 medium containing 10% fetal calf serum. After 6 days, responder spleen cells were collected and assayed in a 4-h ^{51}Cr-release assay[17] on Mo-env-transfected, M-MuLV-infected and uninfected BALB/c-3T3 cells and the M-MuLV-induced tumour cells LSTRA (H–2^d) and MBL2 (H–2^b) at the indicated effector-to-target ratios. Data are expressed as % specific Cr-release[17]. The spontaneous ^{51}Cr-release of all BALB/c-3T3-derived targets was less than 9%, for LSTRA, 21% and for MBL2, 16%.

Initial screening of the G418-resistant clones for expression of gp70 was done by indirect immunofluorescence on ethanol-fixed cells using a goat anti-Raucher gp70 antiserum (given by Dr Argyrios Theofilopoulos) followed by an anti-goat fluorescein isothiocyanate (FITC)-conjugated antiserum. Those clones which appeared positive by indirect immunofluorescence were analysed further.

M-MuLV-specific proteins in the transfected clones were identified by immunoprecipitation and analysis of cellular proteins on 7–15% polyacrylamide gels (Fig. 2). Extracts made from cells which had been metabolically labelled with ^{3}H-leucine were reacted with a goat antiserum which had been raised against Tween–ether-disrupted M-MuLV virions (supplied by the NCI, NIH). This antiserum was able to immunoprecipitate the envelope gene products gp70 and p15E and the core virion proteins p30, p15 and p12 from ^{3}H-labelled virions (see lane 1 of Fig. 2). No M-MuLV-specific proteins were present in uninfected BALB/c-3T3 cells (lane 2 of Fig. 2). gp70, p15E, p30 and pr80 (the precursor to gp70 and p15E) could be immunoprecipitated from M-MuLV-infected BALB/c-3T3 cells (Fig. 2, lane 3). Analysis of the immunoprecipitates of cell extracts prepared from Mo-env-transfected clones N4, E4 and E5 (Fig. 2, lanes 5, 6 and 7, respectively) indicate that these cell lines synthesized pr80, gp70 and p15E. No p30 or any other non-envelope viral protein was observed in these transfected

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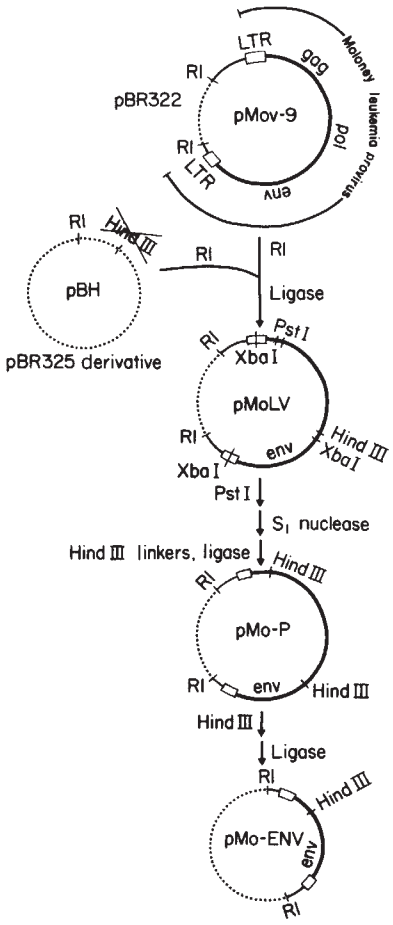

Fig. 1 Construction of a Mo-env expression vector. pMov-9 is a biologically active molecular clone of a proviral M-MuLV genome[7] in pBR322. The provirus was excised using *Eco*RI and cloned into the *Eco*RI site of plasmid pBH. pBH is a derivative of pBR325 (ref. 18) in which the *Hin*dIII site has been ablated by *in vitro* mutagenesis (D.V.F., unpublished). The resulting plasmid, pMoLV, was partially digested with *Pst*I and those fragments which had been cut only once or twice were treated with S_1 nuclease (BRL) to generate blunt ends. *Hin*dIII oligonucleotide linkers were attached with DNA ligase and the plasmid recircularized after partial digestion with *Hin*dIII, generating pMo-P. This plasmid does not express any *gag* or *pol* region products after transfection into mouse cells, presumably because of a deletion in the 5′ portion of the coding sequences for *gag*, generated during its construction. The splice donor and acceptor sites for the *env* gene transcript are intact, however, and allow expression of *env* products on transfection. More efficient expression occurs when the intervening sequences between the viral promoter in the LTR and the start of the *env* gene are removed, resulting in pMo-env. All plasmid transformations were done in $CaCl_2$-shocked *Escherichia coli* HB101 (ref. 19). Colonies were screened by the minilysate technique[20]. Unless otherwise indicated, all enzymes were from New England Biolabs and digestions were done according to the suppliers' recommendations. Transfections were carried out by a modification of the calcium phosphate–DNA precipitate method of Graham and van der Eb[8]. Cells were plated onto a 100-mm dish at 10^6 cells per dish, 24 h before transfection. 30 μg of DNA, including salmon sperm carrier DNA, were dissolved in 1 ml of HBS (21 mM Tris *p*H 6.9, 150 mM NaCl, 0.7 mM Na_2HPO_4); 0.6% vol. of 2.5 M $CaCl_2$ was added and a fine precipitate allowed to form at room temperature for 15 min. This solution was added to each dish of cells and the plates were incubated at room temperature for 20 min, then at 37 °C for 4 h. The cells were washed with Dulbecco's modified Eagle's medium (DMEM), then exposed to 15% glycerol in HBS for 3 min at 37 °C. The glycerol was removed by washing and replaced with DMEM; 48 h later, each plate was trypsinized and replated onto three 100-mm plates. If selective medium was to be used, it was added at this time.

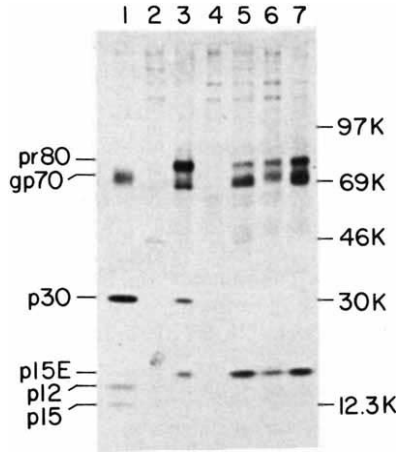

Fig. 2 Analysis of Mo-env expression in transfected BALB/c-3T3 cells. Lane 1, M-MuLV-disrupted virions; lane 2, uninfected BALB/c-3T3 cells; lane 3, BALB/c-3T3 cells infected with M-MuLV; lane 4, clone P4; lane 5, clone N4; lane 6, clone E4; lane 7, clone E5.

Methods: Cell monolayers in 60-mm tissue culture dishes, grown in DMEM supplemented with glutamine (0.03%), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 5% fetal calf serum, were washed twice in phosphate-buffered saline (PBS), *p*H 7.4 and incubated at 37 °C for 1 h in leucine-free modified Eagle's medium (MEM). The medium was removed and the cells were radiolabelled for 2 h at 37 °C by incubation with 1 ml of leucine-free MEM containing 50 μCi ^{3}H-leucine. After labelling, each culture was washed three times in PBS and the cells extracted by adding 1 ml of protein lysis buffer (0.1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.01 M phosphate *p*H 7.5, 0.1 M NaCl). After incubation at 4 °C for 20 min, the extracts were centrifuged at 12,000*g* and the supernatants used to immunoprecipitate proteins. ^{3}H-labelled virus was isolated from the media of M-MuLV-infected cells grown overnight in leucine-free MEM containing 150 μCi ^{3}H-leucine. Virus was pelleted by centrifugation at 128,000*g* and resuspended in 1 ml of protein lysis buffer and extracts were prepared as above. Cellular and viral extracts were reacted for 1.5 h at 4 °C with 10 μl of a goat antiserum raised against Tween–ether-disrupted M-MuLV virions. Immune complexes were precipitated by adding 50 μl of a 10% (w/v) suspension of protein A-bearing *Staphylococcus aureus* (Pansorbin, Calbiochem.) After incubation for 10 min, the suspensions were centrifuged at 12,000*g* for 15 s. The supernatants were discarded and the bacterial pellets washed three times in 150 NaCl–10 mM Tris (*p*H 7.2), containing 1% Nonidet P-40 and 0.5 M LiCl. Immune complexes were eluted by resuspending the bacteria in sample buffer (1% SDS, 1% 2-mercaptoethanol, 50 mM Tris (*p*H 6.8), 5% glycerol) and heating for 3 min at 100 °C. The bacteria were pelleted and the supernatants analysed by SDS-polyacrylamide gel electrophoresis on 7–15% polyacrylamide gradients using the Laemmli buffer system[22]. Gels were fixed for 1 h in 10% trichloroacetic acid, prepared for fluorography using Enhance (NEN) and the dried gels exposed on Kodak X-Omat XR-5 film for 4–6 days at −70 °C.

clones. This indicates that the observed expression of pr80, gp70 and p15E is occurring from translation of the transfected envelope gene and not from expression of endogenous virus. Analysis of the medium in which these cells were growing showed no assayable reverse transcriptase (data not shown). The clone designated P4 (Fig. 2, lane 4) which was transfected with the Mo-env and MSV-neo vectors and was G418-resistant, but was negative in the initial screening by immunofluorescence for gp70, did not express any immunoprecipitable M-MuLV-specific proteins.

Cell surface expression of gp70 by the Mo-env-transfected clones was studied using fluorescence-activated cell sorting (FACS). The transfected cells as well as M-MuLV-infected and uninfected BALB/c-3T3 cells were treated with an anti-Raucher gp70 antiserum followed by an anti-goat FITC-conjugated serum. Figure 3*a* shows the cell surface expression of gp70 observed on M-MuLV-infected cells compared with uninfected BALB/c-3T3 cells. When Mo-env-transfected cells are

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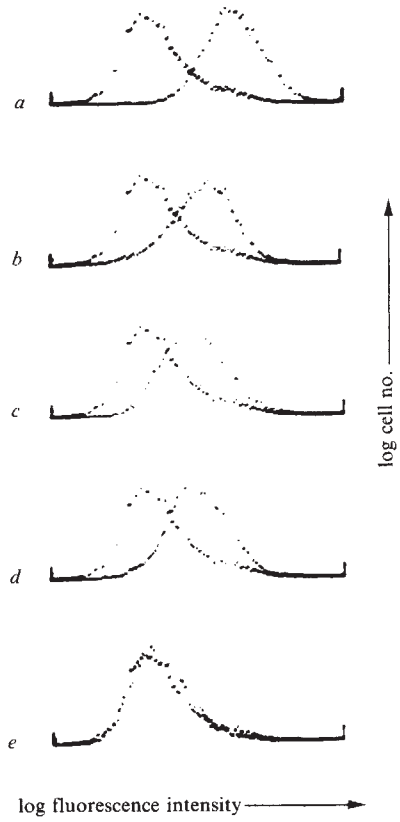

Fig. 3 Detection of gp70 on the surface of Mo-env-transfected cells by FACS analysis. *a*, Uninfected BALB/c-3T3 cells (peak = 92) and M-MuLV-infected BALB/c-3T3 cells (peak = 171); *b*, uninfected BALB/c-3T3 cells and clone N4 (peak = 139); *c*, uninfected BALB/c-3T3 cells and clone E4 (peak = 116); *d*, uninfected BALB/c-3T3 cells and clone E5 (peak = 146); *e*, uninfected BALB/c-3T3 cells and clone P4 (peak = 82). Peak values are arbitrary units of fluorescence intensity.

Methods: Single cell suspensions of uninfected, M-MuLV-infected and Mo-env-transfected cells were prepared by treating cell monolayers with 0.02% Versene (Gibco) at 37 °C for 20 min. The cells were washed three times in PBS, then 3×10^6 cells were resuspended in 100 μl of a 1:50 dilution of goat anti-Raucher gp70 and incubated at 4 °C for 1 h. After washing the cells three times in PBS, they were resuspended in 100 μl of a 1:25 dilution of anti-goat FITC-conjugated antibody (Cappel) and incubated for 1 h at 37 °C. The cells were washed three times more in PBS then fixed in PBS containing 1% paraformaldehyde. At least 10,000 cells were run through the FACS analyser. The data are expressed as log fluorescence intensity.

compared with uninfected cells, clones N4 (Fig 3*b*), E4 (Fig. 3*c*) and E5 (Fig. 3*d*) all show significant levels of gp70 expression on the cell surface. Clone P4 (Fig. 3*e*), which does not express any immunoprecipitable M-MuLV-specific proteins, is indistinguishable from uninfected cells with regard to cell surface gp70. The peak of fluorescence intensity for clones N4, E4 and E5 was calculated as 139, 116 and 146 units, respectively (in arbitrary logarithmic units of fluorescence intensity) compared with 92 units for uninfected cells and 171 units for infected cells. These findings indicate that cell lines which stably express different levels of surface gp70 can be generated by transfection with molecularly cloned M-MuLV *env* gene.

To determine whether the products of the transfected envelope gene were biologically active, transfected clones expressing gp70 were assayed for their susceptibility to superinfection by M-MSV. Because of the phenomenon of 'viral interference'[10], it would be expected that those transfected cell lines producing gp70 would be relatively resistant to superinfection by M-MSV (a sarcoma virus pseudotyped with the envelope proteins of M-MuLV and therefore having the host range of M-MuLV). Accordingly, a stock of M-MSV was titred on uninfected cells, M-MuLV-infected cells and the transfected clones, as described previously[11]. Uninfected BALB/c-3T3 cells and the transfected clone P4 were equally permissive for infection and transformation by M-MSV (2.5×10^4 and 5.0×10^4 focus-forming units (FFU) per ml of virus stock, respectively). Transfected clone E4 showed some resistance to M-MSV infection (titre of 3.7×10^2 FFU ml^{-1}) while clones N4 and E5 were as resistant to infection by M-MSV as the M-MuLV-infected cells (all <25 FFU ml^{-1}). These results are consistent with the FACS analysis of cell surface gp70 expression, in that clone E4 expresses lower levels of gp70 on its surface than do N4 and E5 and is also less resistant to M-MSV superinfection.

Secondary *in vitro* stimulation with M-MuLV-induced tumour cells of spleen cells taken from mice which had previously rejected M-MSV-induced tumours results in the generation of H-2-restricted, M-MSV-specific CTL[12,13]. Using CTL generated in such a manner, Mo-env-transfected cells were analysed for their susceptibility to lysis in a 4-h ^{51}Cr-release assay. The results (Table 1) indicate that those transfected cell lines expressing M-MuLV gp70 on their surface are susceptible to CTL-mediated lysis. The per cent specific ^{51}Cr-release at an effector-to-target ratio of 80:1 for clones N4, E4 and E5 was 34.8%, 38.4% and 56.4%, respectively. This level of lysis was lower than that observed for M-MuLV-infected BALB/c-3T3 cells (78.1%) and the syngeneic M-MuLV-induced tumour cell line, LSTRA (70.5%). No significant lysis was observed with uninfected BALB/c-3T3 cells (7.7%), clone P4 (8.4%) and the allogeneic M-MuLV-induced lymphoma, MBL2 (16.3%).

The data presented here indicate that cell lines can be constructed, via transfection with retroviral genes together with viral promoter and control elements, which express specific viral proteins at levels comparable with those found in productively infected cells. Furthermore, in the case of the *env* gene of M-MuLV, the translated gene products undergo appropriate processing, including cleavage and glycosylation, and appear on the surface of the transfected cells in a biologically active form. Clonal selection of cells from a bulk transfection has yielded individual cell lines which stably express viral gp70 on their surfaces at different levels.

We have shown that transfected BALB/c-3T3 cells which are expressing the single retroviral gene *env* on their surfaces are specifically recognized and lysed by M-MuLV-specific CTL. In addition, the susceptibility of these transfected cells to CTL-mediated lysis appears to be a function of the level of expression of cell surface gp70. Taken together, these data indicate a role for gp70 in the recognition of MuLV-infected cells or MuLV-induced tumours by MuLV-specific CTL. Because all the transfected clones which express gp70 on their surfaces also express the other product of the *env* gene, p15E, it is impossible at this stage to determine whether p15E has a direct role in CTL recognition. Although p15E seems to be very closely associated with the cell membrane and is unavailable to react in extracellular iodination reactions[14], it is clear that an MuLV infection can elicit anti-p15E antibodies[15] and that such antibodies are lytic for infected cells[16]. Studies are underway to examine the role of p15E.

Although the reduced susceptibility of Mo-env-transfected cells to lysis by CTL may be due to their lower level of *env* expression, whether other MuLV-specific proteins also serve as target recognition structures on infected cells or tumours remains to be determined. Our observation that higher effector-to-target ratios are required to produce maximal killing of the gp70-expressing clones than of M-MuLV-infected cells suggests that other viral proteins, for example, glycosylated products of the *gag* region, may also be recognized by virus-specific CTL. Recognition of another viral protein in addition to gp70 would explain the contradictory results obtained in the studies which attempted to examine the role of gp70 in CTL recognition using purified gp70 or anti-gp70 antibodies[4–6].

This work was supported by NIH grants AI-17258 and RR05526, and by training grant P32 CA 09361-02 from the

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National Cancer Institute and the Dyson Foundation. D.V.F. is the recipient of an American Cancer Society Career Development Award. S.J.B. is the recipient of an American Cancer Society Faculty Research Award.

Received 7 June; accepted 16 August 1983.

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Cloning and sequence analysis of calf cDNA and human genomic DNA encoding α-subunit precursor of muscle acetylcholine receptor

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The nicotinic acetylcholine receptor (AChR) from fish electric organ is well characterized and is known to consist of five subunits present in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ (reviewed in refs 1–3). The mammalian skeletal muscle AChR is thought to have a similar subunit structure[4–6]. We have recently elucidated the primary structures of the α-, β-, γ- and δ-subunit precursors of the *Torpedo californica* AChR by cloning and sequencing cDNAs for these polypeptides[7–9]; cDNA sequences for the γ-subunit precursor of the *T. californica* AChR[10] and the α-subunit precursor of the *Torpedo marmorata* AChR[11,12] have also been reported by other groups. The four subunits exhibit conspicuous sequence homology and are similar in hydrophilicity profile and predicted secondary structure, thus being most probably oriented in a pseudosymmetric fashion across the membrane. The transmembrane topology of the subunit molecules and the locations of functionally important regions, such as the acetylcholine binding site and the transmembrane segments which may be involved in the ionic channel, have been proposed. We have now cloned cDNA for the α-subunit precursor of the calf skeletal muscle AChR and a human genomic DNA segment containing the corresponding gene. Nucleotide sequence analysis of the cloned DNAs has revealed the primary structures of the calf and human AChR α-subunit precursors, which exhibit marked sequence homology with their *Torpedo* counterpart. The protein-coding sequence of the human AChR α-subunit precursor gene is divided by eight introns into nine exons, which seem to correspond to different structural and functional domains of the subunit precursor molecule.

A library of cDNA clones derived from poly(A)$^+$ RNA from newborn calf skeletal muscle was constructed with the plasmid DNA vector of Okayama and Berg[13] and was screened by hybridization with a cDNA fragment containing most of the protein-coding sequence for the *T. californica* AChR α-subunit precursor. Because the initial calf cDNA clone thus obtained did not carry the entire protein-coding sequence, additional clones harbouring cDNA for a further upstream region were isolated by screening cDNA transcripts that were formed by reverse transcriptase-mediated elongation of a specific oligodeoxyribonucleotide primer and cloned into plasmid pBR322 (see Fig. 1 legend). A human genomic DNA library[14] was then screened for phage carrying AChR α-subunit precursor gene sequences by hybridization with a calf cDNA probe. Because the initial phage clone obtained did not contain the entire protein-coding sequence, additional clones that harboured a DNA fragment extending further upstream or downstream were isolated by screening the genomic DNA library with a hybridization probe derived from the 5′- or 3′-terminal region of the DNA insert carried by the initial clone (see Fig. 1 legend). Blot hybridization analysis[15] of human placental cellular DNA at 55–60 °C with human genomic DNA and calf cDNA probes exhibited hybridization-positive fragments anticipated from the restriction map of the cloned genomic DNA, thus ensuring that the cloned DNA segment containing the AChR α-subunit precursor gene retains the sequence organization found in the cellular DNA. The cloned calf cDNA as well as the protein-coding segments and their surrounding areas of the cloned human genomic DNA were subjected to nucleotide sequence analysis[16] according to the strategy indicated in Fig. 1.

The calf cDNA and human genomic DNA sequences determined are shown in Fig. 2. Comparison of the two sequences has revealed that the protein-coding sequence of the human AChR α-subunit precursor gene is split by eight introns. The primary structures of the calf and human AChR α-subunit precursors have been deduced from the cDNA and genomic DNA sequences, respectively, by using the reading frame corresponding to the known amino acid sequence of the *T. californica* AChR α-subunit precursor[7] (Fig. 2). The assignment of the translational initiation site (ATG) is supported by the fact that a nonsense codon (TAG) is found in-frame 4–6 nucleotides upstream from this ATG triplet on the human sequence; it seems unlikely that this nonsense codon is eliminated by RNA splicing because the sequence immediately preceding it does not coincide with the consensus sequences for acceptor sites[17]. The 3′-untranslated region of the calf mRNA is unusually long (2,220 nucleotides excluding the poly(A) tract), containing the polyadenylation signal[18] AAUAAA (residues 3,512–3,517 and 3,521–3,526) 15 and 6 nucleotides upstream from the poly(A) tract. The length of the 3′-untranslated region is consistent with the size of this mRNA (~4,500 nucleotides) estimated by blot hybridization analysis of calf skeletal muscle poly(A)$^+$ RNA (Fig. 3). The human genomic DNA sequence shown apparently does not extend to the 5′ end of the exon containing the translational initiation site. It is possible that the gene sequence encoding the 5′-untranslated region is interrupted by one or more introns. The 3′-terminal sequence of the human gene is given for the region where its homology with the calf cDNA sequence is evident. The cloned human DNA segment extending ~11.3 kilobase pairs (kbp) further downstream exhibited no detectable hybridization signals at 55 °C with the 3′-untranslated sequence of the calf cDNA. This suggests either an insufficient degree of homology between the human and calf sequences in this region or the presence of an intron(s) in the human 3′-untranslated sequence.

Comparison of the amino acid sequences of the human and calf AChR α-subunit precursors with their *Torpedo* counterpart shows conspicuous homology among them, although the prepeptide sequence is rather divergent (Fig. 4*A*). The mature α-subunits of the human, calf and *T. californica* AChR are all composed of 437 amino acids, having calculated molecular weights of 49,694, 49,897 and 50,116 (ref. 7), respectively. The

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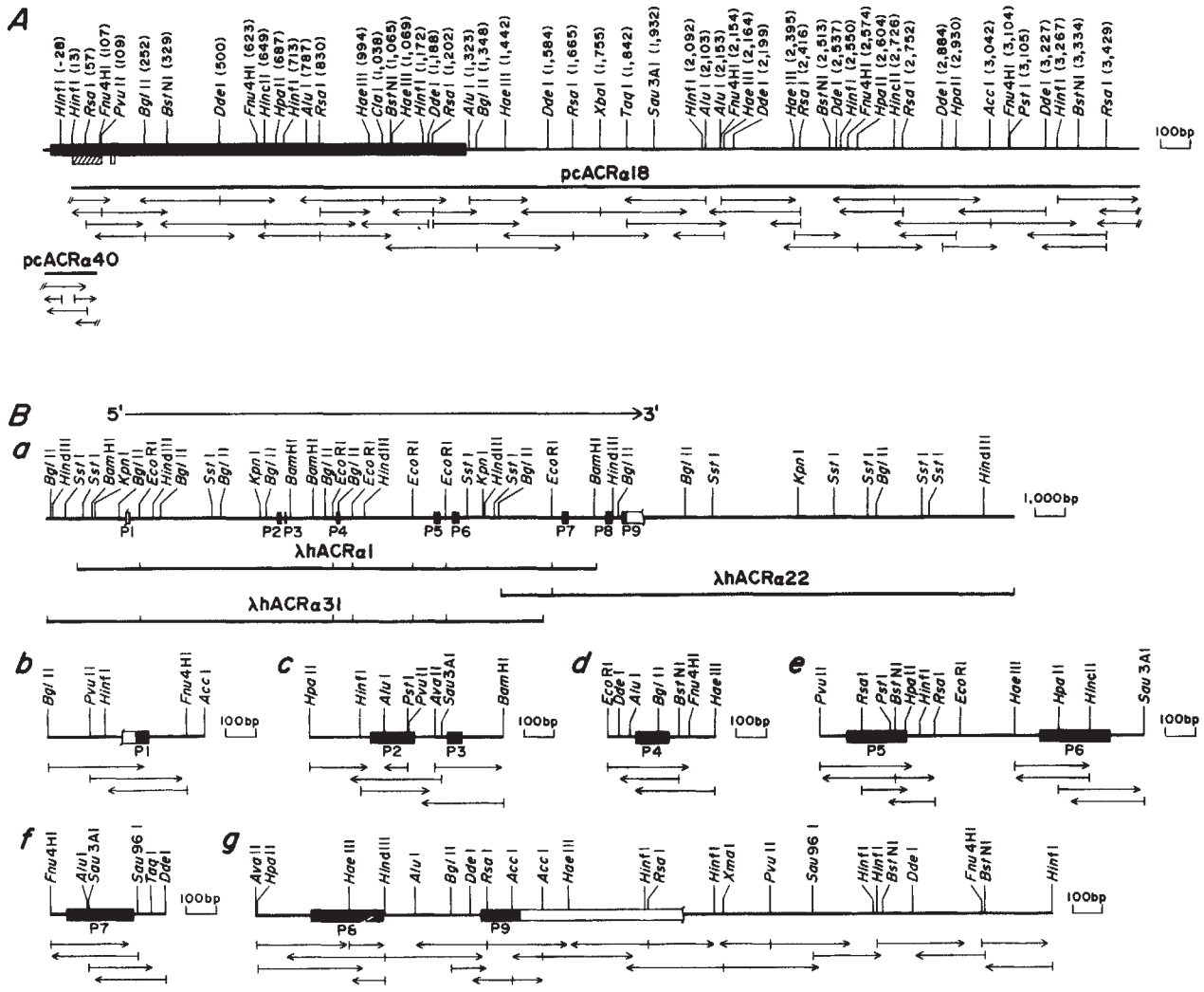

Fig. 1 Restriction mapping and sequencing strategy of cloned calf cDNA (*A*) and cloned human genomic DNA (*B*, *a–g*) encoding the AChR α-subunit precursor. In *A*, only the relevant restriction sites are shown and identified by numbers indicating the 5′-terminal nucleotide generated by cleavage (for the nucleotide numbers, see Fig. 2). The poly(dA) poly(dT) tract and the poly(dG) poly(dC) tails are not included in the restriction map. The protein-coding region is indicated by a closed box, the sequence used for specific priming of reverse transcription by an open box, and the sequence used as hybridization probe for selecting clones by a hatched box. In *B*, the direction of transcription is from left to right. In *a*, all existing sites for the restriction endonucleases indicated are shown, except for the ~1.0-kbp segment located downstream of the 3′-terminal *Hin*dIII site. The *Eco*RI sites in each phage clone, including those derived from the linkers, are indicated by short vertical lines on the line diagram representing the clone. For reference, the locations of the exons (P1–P9) comprising the protein-coding sequence (closed boxes) are indicated (see Fig. 4*B*). In *b–g*, portions of the DNA segment containing the exons are expanded, and only the relevant restriction sites are shown. A scale is given in base pairs (bp) on the right side of each restriction map. The direction and extent of sequence determinations[16] are shown by horizontal arrows under the restriction maps in *A* and *B*, *b–g*; the sites of 5′-end labelling are indicated by short vertical lines at the end of arrows, and the slash marks at the end of arrows mean that the site of 5′-end labelling was located on the vector DNA[13,27] as follows: *Dde*I (left mark) and *Pvu*II (right marks) for pcACRα18; *Pst*I for pcACRα40.

Methods: Total RNA was extracted[28] from newborn calf skeletal muscle, and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography[29]. A cDNA library was constructed by the method of Okayama and Berg[13], using 5.8 μg of poly(A)$^+$ RNA and 2.8 μg of the vector-primer DNA; avian myeloblastosis virus reverse transcriptase was provided by Dr J. W. Beard. *Escherichia coli* χ1776 (ref. 30) or HB101 (ref. 31) was used for transformation[32], and ampicillin-resistant transformants were screened[33] by hybridization at 50 °C with the *Bgl*II(−146)–*Bgl*II(942) fragment (labelled by nick-translation[34] with [α-^{32}P]dCTP) derived from the *T. californica* cDNA clone pACRα30 (ref. 7). One hybridization-positive clone (pcACRα18) was isolated from ~5×10^5 transformants and subjected to nucleotide sequence analysis[16]. The oligodeoxyribonucleotide 5′-GGTTGTCACGATCTG-3′, which is complementary to the mRNA sequence of residues 142–156 (Fig. 2) in clone pcACRα18, was synthesized by the triester method[35] and used for specific priming of the reverse transcription of the calf AChR α-subunit precursor mRNA. Single-stranded cDNA was synthesized using this primer and calf muscle poly(A)$^+$ RNA as template and was converted to double-stranded cDNA, which was cloned in plasmid pBR322 by inserting it into the *Pst*I site using poly(dG) poly(dC) homopolymeric extensions; the procedures used were as described previously[36]. The cDNA clones obtained were screened by hybridization at 60 °C with the *Hin*fI(13)–*Pvu*II(109) fragment (labelled by nick-translation) derived from the 5′-terminal region of clone pcACRα18. Twenty hybridization-positive clones were isolated from ~3×10^5 transformants. One of them (pcACRα40), which apparently harboured the largest cDNA insert, was subjected to DNA sequencing[16]. This clone shared a sequence of 79 bp with clone pcACRα18. The human genomic DNA library used was kindly provided by Dr T. Maniatis and was a collection of recombinant phage that carried human fetal liver DNA fragments generated by partial digestion with *Hae*III and *Alu*I and joined to the λ Charon 4A arms with *Eco*RI linkers[14]. This library was screened[37] by hybridization at 60 °C with the *Bgl*II(252)–*Bgl*II(1,348) fragment excised from the cDNA clone pcACRα18 (labelled by nick-translation). One hybridization-positive phage clone (λhACRα1) was isolated from ~10^6 phage by repeated plaque purification. This clone carried a DNA insert containing seven *Eco*RI fragments with approximate lengths of 2.1, 6.4, 0.6, 2.0, 1.1, 3.5 and 1.5 kbp in the 5′ to 3′ direction. Subsequent screening[37] of ~10^6 plaques by hybridization at 60 °C with the 3′-terminal 1.5-kbp *Eco*RI fragment derived from clone λhACRα1 (labelled by nick-translation) yielded three hybridization-positive clones. One of them carried the same DNA insert as clone λhACRα1, and the remaining two (represented by clone λhACRα22) harboured a DNA insert containing two *Eco*RI fragments with approximate lengths of 1.7 and 15.3 kbp in the 5′ to 3′ direction. Further screening[37] of ~10^6 plaques by hybridization at 60 °C with the ~0.5-kbp *Eco*RI–*Hin*dIII fragment derived from the 6.4-kbp *Eco*RI fragment of clone λhACRα1 (labelled by nick-translation) yielded one hybridization-positive clone (λhACRα31), which carried a DNA insert containing six *Eco*RI fragments with approximate lengths of 3.1, 6.4, 0.6, 2.0, 1.1 and 3.2 kbp in the 5′ to 3′ direction. The DNA segments carried by clones λhACRα1, λhACRα22 and λhACRα31 overlap one another as shown in *B*, *a*. All genomic *Eco*RI fragments that hybridized with the cDNA clone pcACRα18 or pcACRα40 were subcloned in plasmid pBR322 for restriction mapping and nucleotide sequence analysis[16], except that the 15.3-kbp *Eco*RI fragment in clone λhACRα22 was cleaved by *Hin*dIII prior to subcloning.

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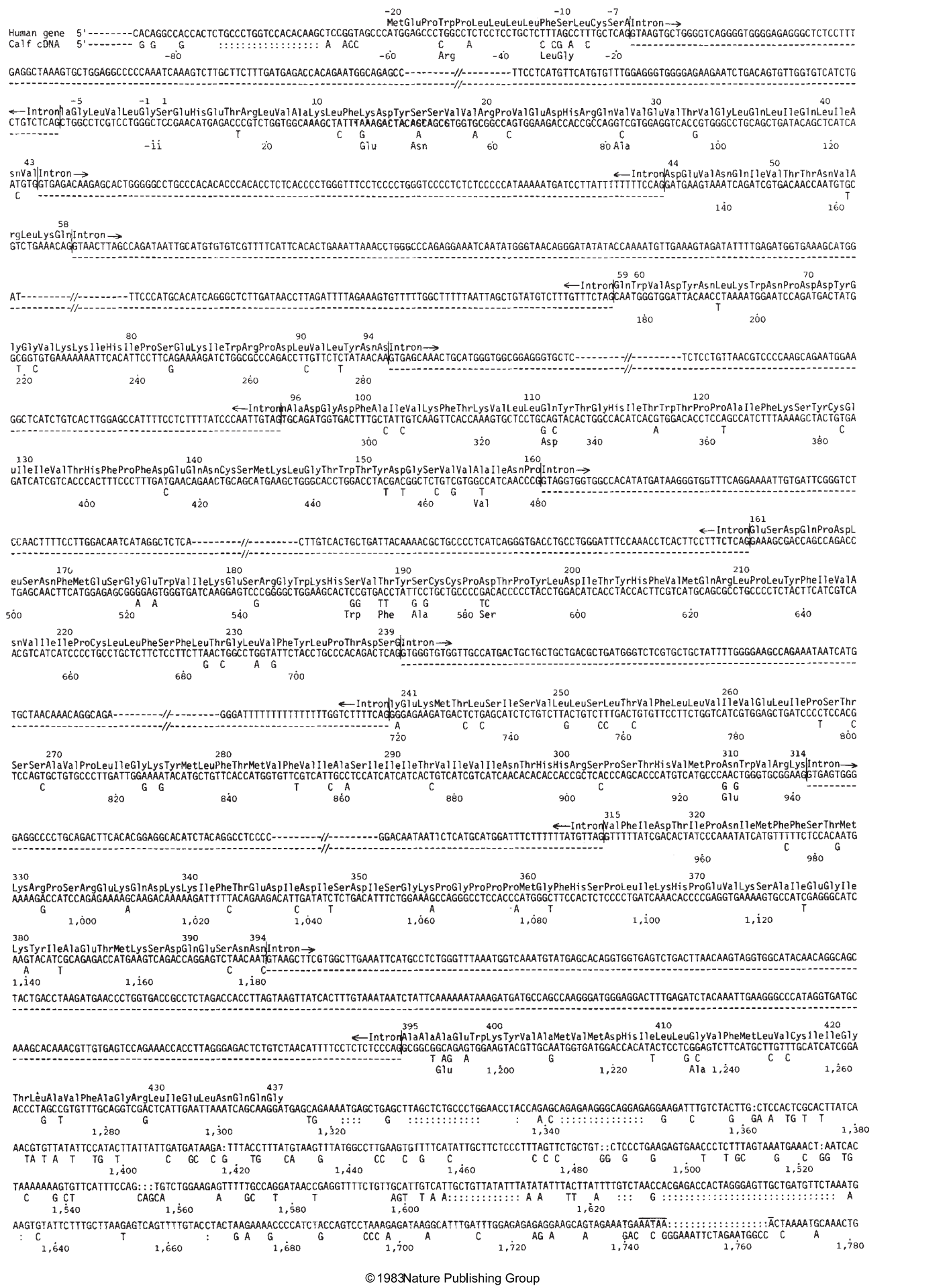

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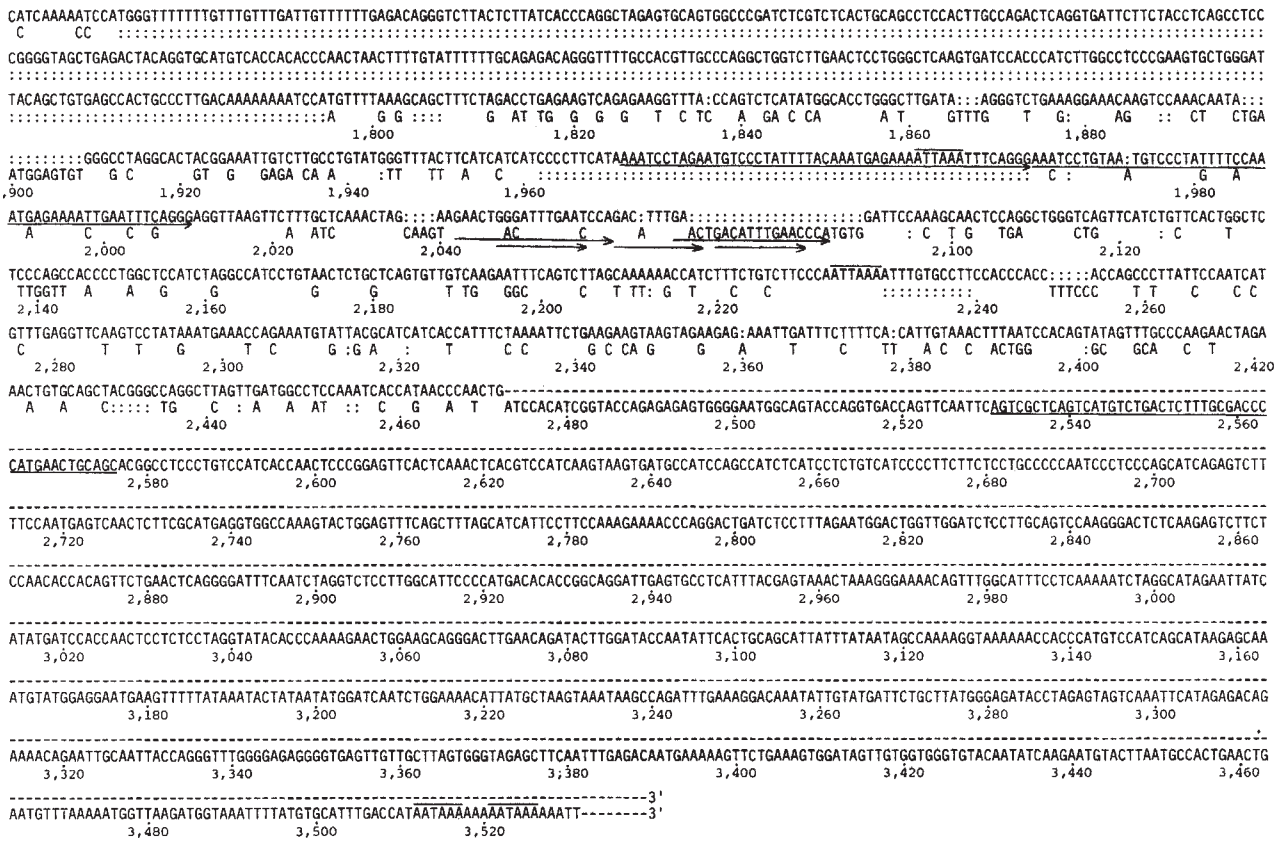

Fig. 2 Nucleotide sequences of the human genomic DNA (upper line) and the calf cDNA (lower line) encoding the AChR α-subunit precursor. The nucleotide sequence of the human message strand, together with the deduced amino acid sequence, is shown, and the nucleotide and amino acid differences found in the calf sequences aligned are displayed beneath the human sequences; the absence of a nucleotide or an amino acid in the calf sequence indicates that the human and calf sequences are the same; the presence of a colon in either nucleotide sequence indicates a gap; the hyphens indicate sequences that have not been determined or cloned or that exhibit no evident homology with their counterpart. Amino acid residues are numbered beginning with the amino-terminal residue of the mature α-subunit, and the preceding residues are indicated by negative numbers. Nucleotide residues in the calf cDNA are numbered in the 5′ to 3′ direction, beginning with the first residue of the codon specifying the amino-terminal residue of the mature α-subunit, and the nucleotides on the 5′ side of residue 1 are indicated by negative numbers. The exon–intron junctions, which are positioned according to the G-T/A-G rule[38], are indicated by vertical lines. The 5′-terminal sequences shown do not extend to the 5′ end of the mRNA or the gene. The 3′-terminal cDNA sequence presented is followed by a poly(A) tract of 66 nucleotides connected with the vector DNA sequence[13,27], thus apparently representing the complete sequence of this region, whereas the 3′-terminal gene sequence shown is probably incomplete (see text). The polyadenylation signals AATAAA and ATTAAA (refs 18, 39) present downstream of the translational termination site are overlined. The sequence homologous with the bovine repetitive DNA sequences (complementary to a portion of the consensus sequence given in ref. 26) and the direct repeats present in the 3′-untranslated region are shown by an underline and by horizontal arrows beneath the sequences, respectively.

degree of homology between the mature α-subunits is 97, 80 and 81% for the human/calf, human/*Torpedo* and calf/*Torpedo* pairs, respectively. The human and calf AChR α-subunits contain the two cysteine residues (residues 128 and 142) that are conserved in all four subunits of the *T. californica* AChR[7–10] and that have been proposed[7–9] as taking part in the disulphide bridge near the acetylcholine binding site[1,19]. The asparagine residue[7–10] (residue 141) next to one of these cysteine residues is also conserved in the mammalian AChR α-subunits and represents the only possible site of *N*-glycosidic linkage[20]. The four putative transmembrane segments M1–M4, which are present in all four subunits of the *T. californica* AChR and may be involved in the ionic channel[7–10], are similarly found in the mammalian AChR α-subunits. The hydrophilicity profiles[21] as well as the secondary structures[22] predicted from the amino acid sequences of the mammalian AChR α-subunits are also similar to those predicted for the *T. californica* AChR subunits[7–10]. These findings suggest that the mammalian AChR α-subunits exhibit the same transmembrane topology as proposed for the four subunits of the *Torpedo* AChR[7–10,12].

On the basis of the hydrophilicity profile and the transmembrane topology, we have previously proposed[7,9] that the segment of the *T. californica* AChR α-subunit composed of residues 161–166 is the most likely candidate for the 'main immunogenic region' (refs 23–25). The corresponding segment of the mammalian AChR α-subunits has a glutamine residue instead of the arginine residue at position 164.

The protein-coding sequence of the human AChR α-subunit precursor gene is divided into nine exons, designated as P1 to P9 in the 5′ to 3′ direction, as schematically shown in Fig. 4*B*; the 5′ end of exon P1 and the 3′ end of exon P9, which lie beyond the protein-coding region, have not been determined. The lengths of the eight introns are ~4.9 kbp, 111 base pairs (bp), ~1.7 kbp, ~3.1 kbp, ~0.4 kbp, ~3.4 kbp, ~1.2 kbp and 324 bp in the 5′ to 3′ direction. The protein regions encoded by distinct exons seem to correspond to different structural and functional domains of the subunit precursor molecule. Most of the prepeptide is encoded by exon P1, the putative acetylcholine binding region by exon P5, the putative transmembrane segment M1 by exon P6, M2 and M3 by exon P7, M4 by exon P9, and the cytoplasmic region by exon P8. The gene sequence encoding the remaining amino-terminal extracellular region and a small portion of the prepeptide is divided into three exons (P2, P3 and P4). The protein regions corresponding to these three exons may represent as yet unknown structural or functional domains.

Figure 4*B* also shows the degree of amino acid sequence homology in the different protein regions corresponding to the nine exons observed among the AChR α-subunit precursors of the three species. Comparison between the mammalian and *Torpedo* sequences reveals that all of the protein regions, except that constituting most of the prepeptide (exon P1), are highly conserved. The regions encompassing the putative acetylcholine binding site (exon P5) and the putative transmembrane segments

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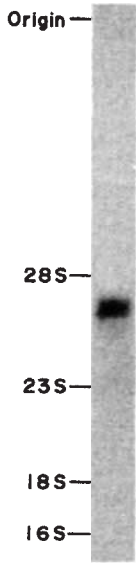


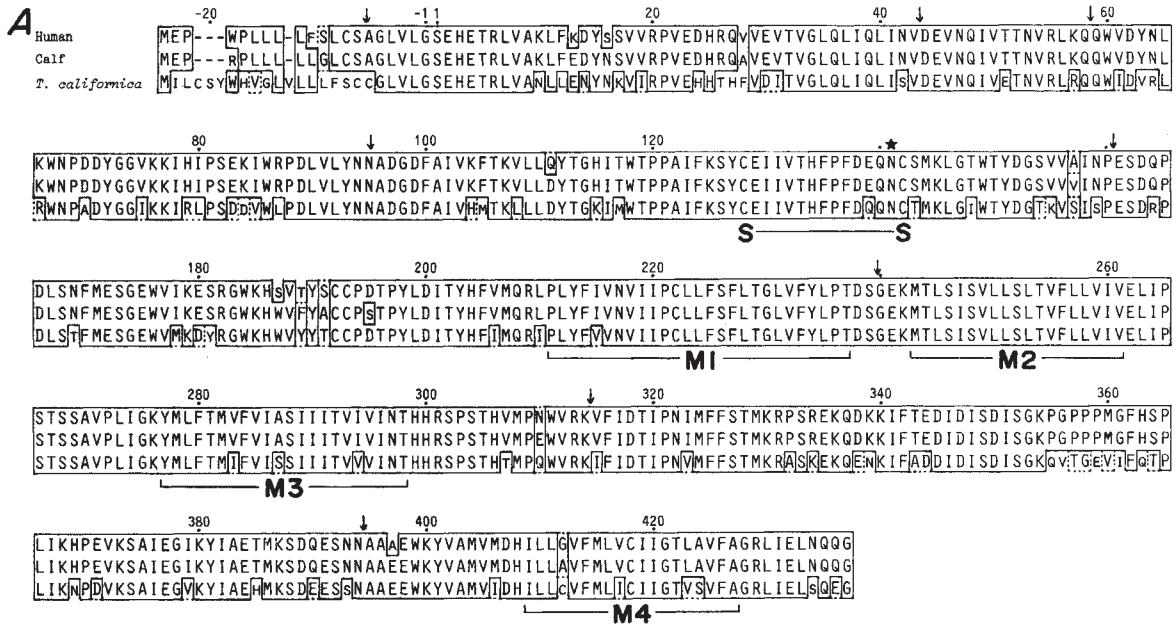
Fig. 3 Autoradiogram of blot hybridization analysis of calf skeletal muscle RNA with a cDNA probe. Poly(A)$^+$ RNA (20 μg) was denatured with 1 M glyoxal and 50% dimethyl sulphoxide[40], electrophoresed on a 1.5% agarose gel and transferred to diazobenzyloxymethyl paper[41]. The hybridization probe used was the *Bgl*II(252)–*Bgl*II(1,348) fragment derived from clone pcACRα18; the probe was labelled by nick-translation[34] with [α-^{32}P]dCTP. The size markers used were calf and *E. coli* rRNA[40].

(exons P6, P7 and P9) as well as the region comprising amino acid residues 44–58 (exon P3) are particularly well conserved.

A peculiar feature of the 3′-untranslated region of the calf AChR α-subunit precursor mRNA is that it contains a 46 nucleotide long sequence (residues 2,531–2,576) homologous with the interspersed repetitive DNA sequences present in the bovine genome[26]. Note also that direct repeats of 19 nucleotides (residues 2,042–2,060 and 2,068–2,086) and 11 nucleotides (residues 2,047–2,057, 2,061–2,071 and 2,073–2,083) are present in the 3′-untranslated region of this mRNA. The human AChR α-subunit precursor gene also contains direct repeats of 49 nucleotides (with four mismatches) in the corresponding region (see Fig. 2).

Recently, the amino-terminal 35 amino acids of the fetal calf muscle AChR α-subunit have been sequenced[6]. The corresponding amino acid sequence deduced from our cDNA sequence agrees with these data except for residues 17, 26, 27, 30 and 32.

We thank Dr Paul Berg and Dr Hiroto Okayama for providing us with their high-efficiency cloning system, Dr Tom Maniatis for the human genomic DNA library, and Dr Takashi Miyata for computer analysis. This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan, the Mitsubishi Foundation and the Japanese Foundation of Metabolism and Diseases.



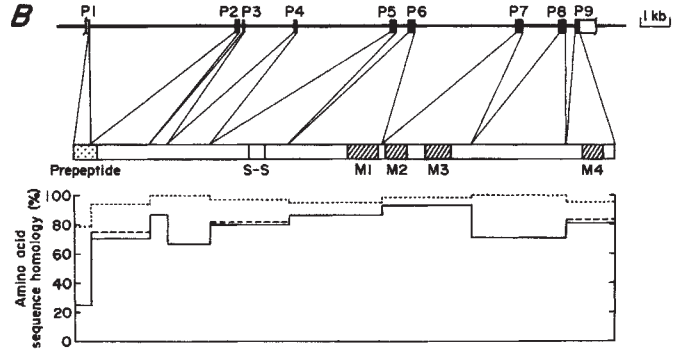


Fig. 4 Alignment of the amino acid sequences of the human, calf and *T. californica* AChR α-subunit precursors (*A*) and amino acid sequence homology in the different protein regions encoded by the nine exons (*B*). In *A*, the human (top), calf (middle) and *T. californica* (bottom) sequences are shown by the one-letter amino acid notation. The *T. californica* sequence has been taken from ref. 7. Large letters represent sets of identical residues (enclosed with solid lines) and sets of residues considered to be favoured amino acid substitutions (enclosed with dotted lines). Small letters represent the remaining residues. Favoured amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E and Q; H, R and K; M, I, L and V; F, Y and W (ref. 42). Gaps (–) have been inserted in the prepeptide region to achieve maximum homology. Amino acid residues are numbered beginning with the amino-terminal residue of the mature α-subunit; the positions in the aligned prepeptide sequences including gaps are indicated by negative numbers. The positions at which the protein-coding sequence is interrupted by introns are indicated by arrows. The asparagine residue as possible glycosylation site is marked with an asterisk. In *B*, the exons (P1–P9) comprising the protein-coding sequence (closed boxes) are shown on the upper line; the lengths of exon P1 and exon P9, which extend beyond the protein-coding region, have not been determined; a scale is given on the right side. The protein regions encoded by the respective exons are shown in the middle diagram. The degree of amino acid sequence homology in each of these protein regions is given below for the human/calf (dotted lines), human/*Torpedo* (dashed lines or solid lines where no dashed lines are shown) pairs; the amino acid residues whose codons are split by introns have not been included in the calculation of homology, and gaps have been counted as one substitution regardless of their length. The positions of the prepeptide (stippled in *B*), the putative disulphide bridge proposed as being in close proximity to the acetylcholine binding site (S–S) and the putative transmembrane segments M1–M4 (hatched in *B*) are indicated.

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Received 27 June; accepted 23 August 1983.

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A DNA insertion in the apolipoprotein A-I gene of patients with premature atherosclerosis

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Apolipoprotein A-I (apo A-I) is the major protein constituent of high-density lipoprotein (HDL)[1]. The study of the apo A-I gene is of interest because plasma levels of HDL have been inversely correlated with the development of coronary artery disease[2] and because polymorphisms related to this gene have been associated with hypertriglyceridaemia[3] and premature atherosclerosis[4,5]. We have recently isolated and characterized the human apo A-I gene[6] and have shown that apo A-I and apoliprotein C-III (apo C-III) genes are physically linked[7] and that a polymorphism (of unknown frequency in the general population) of the apo A-I gene is inherited as a mendelian trait linked to premature atherosclerosis in an affected family[5] (not the same polymorphism as has previously been reported to be associated with hypertriglyceridaemia). Here we report that this polymorphism is due an at least 6.5-kilobase (kb) DNA insertion in the coding region of the apo A-I gene and that there is no detectable alteration (as determined by limited genomic blotting analysis) of the apo C-III gene of these patients.

Chromosomal DNA was prepared from the blood of patients with premature atherosclerosis[5] and used for genomic blotting analyses with radioactively labelled probes containing sequences derived from the normal apo A-I or apo C-III genes. Preliminary studies using the apo A-I cDNA derived[8] pAI-113 probe (Fig. 1*A'*) showed that *Eco*RI-, *Bam*HI- or *Pst*I-digested DNA from apo A-I–apo C-III-deficient patients produces hybridization bands of different size from those obtained from similarly digested DNA from normal individuals[5] (Table 1). These results indicated that this difference is not due to alteration of a single restriction site, and implied that it may be due to a DNA deletion or insertion in the apo A-I gene locus of these patients. In addition, this defect was shown[5] to involve the restriction site *Hin*dIII present in the second intervening sequence (IVS2) of the apo A-I gene (Fig. 1*A'*). Therefore, if a DNA deletion occurred in the apo A-I gene of these patients, it should extend 5′ of the genomic sequences corresponding to the pAI-113 probe and should include the *Hin*dIII site present in IVS2. The size of such a deletion should be at least 6.5 kb, as indicated by the difference (13 bases–6.5 kb) between the sizes of the *Eco*RI restriction bands obtained by genomic blotting analysis of DNA prepared from normal and apo A-I–apo C-III-deficient patients (Table 1). Thus, if the apo A-I gene defect is due to a DNA deletion, it would be expected that molecular probes containing DNA sequences occurring in the 5′ direction of the *Hin*dIII site present in IVS2 (upstream probes) would not produce hybridization bands when used for genomic blotting analysis of DNA prepared from these patients.

A probe containing the normal genomic DNA sequence from the *Hin*dIII site in IVS2 to a *Pst*I site present 0.45 kb upstream of this *Hin*dIII site (upstream probe, see Fig. 1*A'*) was used for genomic blotting analysis of chromosomal DNA prepared from these patients. The resulting autoradiogram (Fig. 1*B*) indicates that the DNA sequences corresponding to this upstream probe have not been eliminated from the genome of apo A-I–apo C-III-deficient patients and are present in genomic restriction fragments, the sizes of which have been compiled in Table 1. These upstream probe DNA sequences are located on 3 or 0.45 kb, *Hin*dIII or *Hin*dIII plus *Pst*I restriction fragments, respectively, in the genome of both normal (Fig. 1*A*, Table 1) and apo A-I–apo C-III-deficient individuals (Fig. 1*B*, Table 1). These results indicate that the apo A-I gene polymorphism observed in apo A-I–apo C-III-deficient patients is due to a DNA insertion which has occurred in the 3′ direction of the *Hin*dIII site present in IVS2 and the 5′ direction of the genomic sequence corresponding to the pAI-113 probe.

The hybridization signals from the nitrocellulose filter used to produce Fig. 1*B* were removed by boiling for 5 min in TE buffer (10 mM Tris, 1 mM EDTA *p*H 7.5), and the filter was rehybridized with a cDNA probe[6] containing the DNA sequences present 100 base pairs (bp) upstream and 312 bp downstream of IVS3 (pAI-101 probe, see Fig. 1*A'*). The resulting autoradiogram (Fig. 1*C*) shows that *Eco*RI, *Bam*HI, *Pst*I, *Pst*I plus *Eco*RI, and *Pst*I plus *Bam*HI digestions of DNA prepared from apo A-I–apo C-III-deficient patients produce, after blotting and hybridization with this pAI-101 probe, two hybridization bands, the sizes of which have been compiled in Table 1. This pattern is a composite of that obtained with the pAI-113 and upstream probes separately (Table 1), and indicates that the DNA insertion in the apo A-I gene of these patients has occurred in the gene region extending from 100 bp upstream to 312 bp downstream of IVS3.

To localize further the site of the apo A-I gene at which this insertion has occurred, chromosomal DNA from these patients was digested with various restriction enzymes, blotted and hybridized with a genomic probe containing the DNA sequence

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spanning IVS3 and extending from 120 bp upstream to 120 bp downstream of this intervening sequence in the normal apo A-I gene (*Dde*I probe, Fig. 1*A'*). The resulting autoradiogram (Fig. 1*D*) shows that *Eco*RI, *Bam*HI, *Pst*I, *Pst*I plus *Eco*RI or *Pst*I plus *Bam*HI digestion of chromosomal DNA from these patients produces the same size (Table 1) bands after blotting and hybridization with either upstream (Fig. 1*B*) or *Dde*I probes (Fig. 1*D*). These results indicate that the DNA insertion found in the apo A-I gene of these patients has occurred on the 3′ side of the genomic sequences corresponding to the sequences present in the *Dde*I probe (Fig. 1*A*, *A'*) and localizes the insertion in the coding region of apo A-I gene.

Finally, limited genomic blotting analysis of the apo C-III gene of these patients was carried out by removing the hybridization signal from the nitrocellulose filter used to produce Fig. 1*D*, followed by rehybridization of this filter with an apo C-III cDNA probe[7] (pCIII-606 probe, Fig. 1*A*). The resulting autoradiogram (Fig. 1*E*) shows that *Pst*I, *Pst*I plus *Bam*HI, or *Pst*I plus *Eco*RI digestion of chromosomal DNA prepared from these patients, after blotting and hybridization with this pCIII-606 probe, produces the same size bands as those found after a similar analysis of normal human DNA (Table 1). These results indicate that there is no gross DNA rearrangement in the region of the apo C-III gene corresponding to the pCIII-606 probe in these apo A-I–apo C-III-deficient patients.

These data (Table 1) allowed the construction of a map of the apo A-I–apo C-III gene complex of these patients (Fig. 2). Figure 2 also shows a partial restriction map of the 5′ and 3′ ends of the insertion found in the apo A-I gene of the apo A-I–apo C-III-deficient patients (Fig. 2, shaded boxes). The sum of the sizes of *Bam*HI hybridization bands obtained with pAI-113 and upstream probes is 18.5 kb when chromosomal DNA from these patients is used (Table 1). Similar analysis of normal DNA produced a 12-kb hybridization band with either pAI-113 or upstream probes (Table 1). It can therefore be estimated that the DNA insertion found in the apo A-I gene of the apo A-I–apo C-III-deficient patients is ~6.5 kb. This estimate is in agreement with the estimate obtained with different restriction enzymes and probes indicated in Table 1. Note, however, that this is a minimum estimate because it is possible that additional *Bam*HI sites are present in the central region (Fig. 2) of this DNA element. Cloning and isolation of this DNA

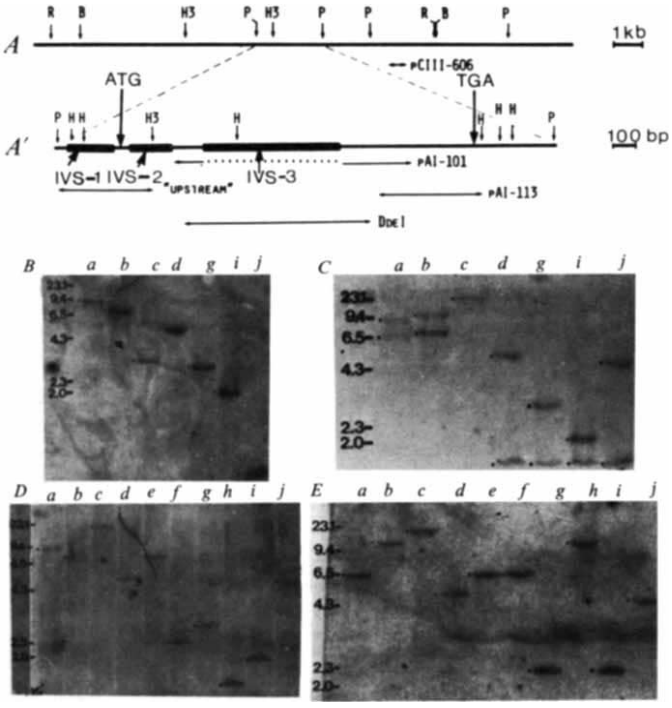

Fig. 1 Genomic blotting analysis of chromosomal DNA prepared from apo A-I–apo C-III-deficient patients. *A*, Restriction map of the apo A-I–apo C-III gene complex in normal individuals. *A'*, 'Blow-up' of the region containing the apo A-I gene. Initiation and termination codons of apo A-I mRNA are shown. Intervening sequences (IVS) in the apo A-I gene (thick lines) are indicated. Double-arrowed lines under the map show the DNA sequences present in the various DNA probes; the dotted line indicates that the pAI-101 probe does not contain intronic DNA sequences. Restriction sites: *Eco*RI (R), *Bam*HI (B), *Pst*I (*P*), *Hin*dIII (H3), *Hpa*II (H). *B–E*, Chromosomal DNA prepared from the blood[5] of apo A-I–apo C-III-deficient patients digested with various restriction enzymes, electrophoresed on 1% agarose gels, blotted and hybridized with the 'upstream' (*B*), pAI-101 (*C*), *Dde*I (*D*) and pCIII-606 (*E*) probes. The resulting autoradiograms are shown. Enzymatic digestions in the gel lanes are as follows: *Eco*RI (*a*), *Bam*HI (*b*), *Hin*dIII (*c*), *Pst*I (*d*), *Eco*RI plus *Bam*HI (*e*), *Eco*RI plus *Hin*dIII (*f*), *Eco*RI plus *Pst*I (*g*), *Bam*HI plus *Hin*dIII (*h*), *Bam*HI plus *Pst*I (*i*), and *Hin*dIII plus *Pst*I (*j*). Size markers are *Hin*dIII-digested λ DNA (Biolabs).

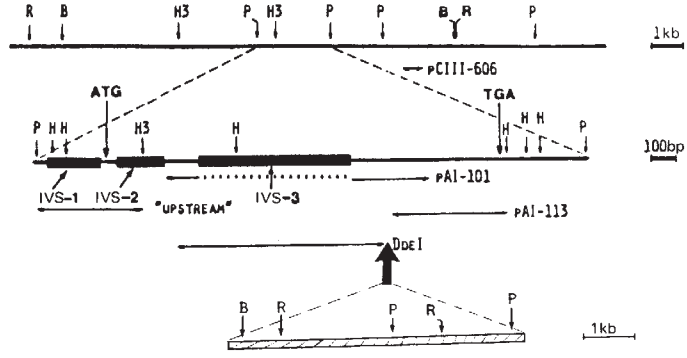

Fig. 2 Restriction map of the apo A-I–apo C-III gene complex in the apo A-I–apo C-III–deficient patients. A restriction map of the normal apo A-I–apo C-III gene complex is indicated as in Fig. 1*A* and *A'*. The site in the apo A-I gene at which the DNA insertion has occurred in these patients is indicated by a thick arrow. Partial restriction map of this insertion (cross-hatched box) is shown below the apo A-I gene map.

Table 1 Sizes (kb) of genomic blotting bands obtained with DNA from normal individuals and apo A-I–apo C-III-deficient patients using various probes

Digestions	pAI-113 probe*		'Upstream probe'		pAI-101 probe		*Dde*I probe		pCIII-606 probe	
	Normal	Patient	Normal†	Patient	Normal†	Patient	Normal†	Patient	Normal‡	Patient
*Eco*RI	13.0	6.5	13.0	9.0	13.0	6.5, 9.0	13.0	9.0	13.0	6.5
*Bam*HI	12.0	11.0	12.0	7.5	12.0	11.0, 7.5	12.0	7.5	12.0	11.0
*Hin*dIII	15.0	23.0	3.0	3.0	15.0	23.0	15.0	23.0	15.0	23.0
*Pst*I	2.2	0.95	2.2	5.0	2.2	0.95, 5.0	2.2	5.0	5.0	5.0
*Pst*I + *Eco*RI	2.2	0.95	2.2	2.8	2.2	0.95, 2.8	2.2	2.8	2.3	2.3
*Pst*I + *Bam*HI	2.2	0.95	2.2	2.0	2.2	0.95, 2.0	2.2	2.0	2.3	2.3
*Pst*I + *Hin*dIII	1.7	0.95	0.45	0.45	1.7	0.95, 4.5	1.7	4.5	5.0	5.0
*Eco*RI + *Bam*HI	12.0	6.5	—	—	—	—	12.0	7.5	12.0	6.5
*Eco*RI + *Hin*dIII	5.5	6.5	—	—	—	—	5.5	2.3	5.5	6.5
*Bam*HI + *Hin*dIII	5.5	11.0	—	—	—	—	5.5	1.4	5.5	11.0

* From refs 5, 7. † Data not shown. ‡ From ref. 6.

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insertion should provide a more accurate size estimation and restriction map.

We demonstrated previously that the restriction site polymorphism of the apo A-I–apo C-III-deficient patients, now shown to be due to a DNA insertion in the apo A-I gene, segregated as an autosomal allele at a single genetic locus among the first degree relatives of these patients[5]. The finding that this DNA insertion was transmitted apparently unchanged through three generations of this kindred indicates that it has been fixed in the germ line of the ancestors of these patients. However, the mechanism responsible for the generation and fixation of this DNA insertion is unknown. Finally, DNA insertions have been shown to interfere with normal gene expression in mouse mutant hybridoma cell lines defective in κ light-chain synthesis[9], *Drosophila* cuticle protein 3 (CP3) mutants[10] and regulatory mutants of the maize *Adnl* gene[11]. If and how the DNA insertion in the apo A-I gene of the apo A-I–apo C-III-deficient patients interferes with apo A-I and possibly apo C-III gene expression remains to be established.

This work was supported by grants from the NIH (HL15895) and the March of Dimes Birth Defects Foundation. J.L. and V.I.Z. are Established Investigators of the American Heart Association. We thank Lorraine M. Duda for assistance.

Received 13 June; accepted 19 August 1983.

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Detection of neuronal tissue from brain grafts with anti-Thy-1.1 antibody

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Grafting of neural tissue has been used to investigate the ability of central nervous system (CNS) axons to elongate into peripheral nerve grafts[1,2]; to investigate the growth characteristics of material transplanted within the CNS[3,4]; and to determine if grafts producing amines or peptides can replace a natural[5,6] or experimentally induced depletion[7,8] of these biogenic molecules. In such experiments donor tissue has been distinguished from host by injection into graft cells of horseradish peroxidase[1,2] or radioactive leucine[9] or by detecting biogenic molecules in the grafted tissue with antibodies[6–8]. All these methods have limitations and a technique which allows identification of the total neuronal cell output from transplanted tissue would be a powerful tool in delineating host–graft interactions. Polymorphic cell-surface antigens have not previously been exploited as markers. The Thy-1 antigen is an abundant glycoprotein of neurones and in the mouse exists in two allotypic forms called Thy-1.1 and Thy-1.2. We now show that tissue from Thy-1.1-positive animals grafted into Thy-1.1-negative brains can be clearly identified in cryostat sections using an anti-Thy-1.1 monoclonal antibody conjugated with peroxidase. We have used the method to study grafts from normal fetal mice which correct a developmental deficiency when transplanted into *hpg* mutant mice.

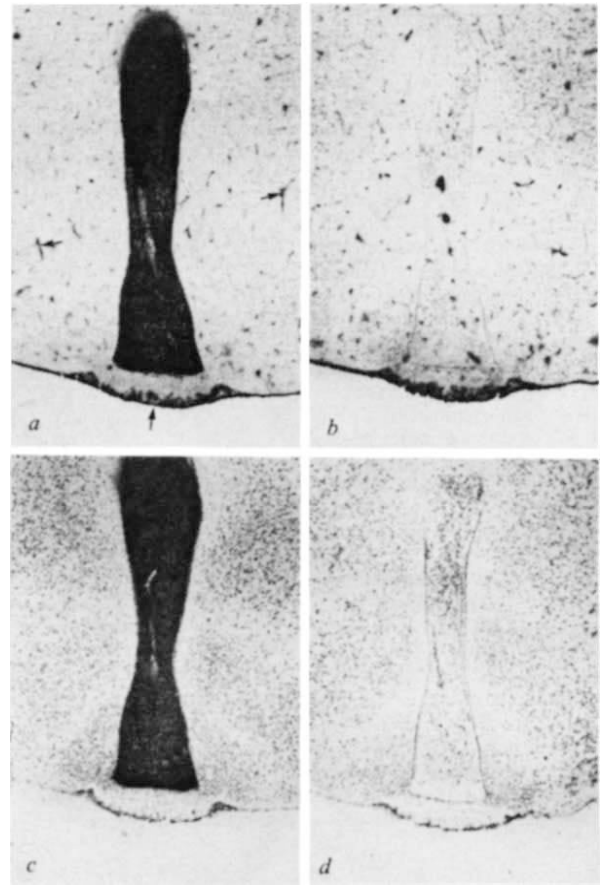

Fig. 1 Localization of Thy-1.1 antigen on grafts of fetal AKR brain in *hpg* recipient brain by immunoperoxidase staining of cryostat sections. *a*, Indirect immunoperoxidase staining with MRC OX-7 antibody shows clear labelling of the graft but also some patchy staining in all areas (see arrows). *b*, Indirect immunoperoxidase control with MRC OX-21 antibody that does not react with rat tissues[24] shows no extensive staining of the graft. Some patchy staining was seen and is probably due to IgG in blood vessels and on surfaces. *c*, Direct immunoperoxidase labelling using peroxidase-conjugated MRC OX-7 antibody shows strong labelling of the graft with no labelling of the recipient. *d*, Control direct immunoperoxidase method using peroxidase-conjugated MRC OX-7 antibody preincubated with purified Thy-1 antigen shows no peroxidase staining and only the counterstain is visible.
Methods: AKR fetal brain was transplanted into the third ventricle of female *hpg* mice as described in Fig. 2 legend. After 14–60 days the brain was removed, and cryostat sections (15 μm) were cut, fixed and stained by an indirect immunoperoxidase method using peroxidase-labelled Fab rabbit anti-mouse IgG as in ref. 25. For direct immunoperoxidase labelling $F(ab')_2$ MRC OX-7 was prepared[17] and conjugated to horseradish peroxidase as in ref. 25. Sections were stained with this reagent at 5 μg ml^{-1} for 1 h at 4 °C. For the control (*d*) the MRC OX-7 peroxidase conjugate was preincubated for 30 min at 4 °C with 20 μg ml^{-1} purified rat thymocyte Thy-1 (ref. 11) before staining of tissue sections. Sections were counterstained with toluidine blue. (×100).

The Thy-1 antigen[10,11] is widely found on the surface membranes of cell bodies and processes of central and peripheral neurones[12,13]. The molecule has been estimated to constitute up to 7.5% of the surface protein of axons[14]. The only mature neurones thought to be Thy-1-negative are found in the retina[15]. Thy-1 is barely detectable in neonatal brain, and expression begins to rise at about day 7 and approaches adult levels by about day 20 (ref. 10). In the mouse, the polypeptide part of the Thy-1 glycoprotein consists of 112 amino acids, and the two allotypic forms differ by one amino acid at residue 89, where Thy-1.1 has arginine and Thy-1.2 glutamine[16]. Monoclonal IgG antibodies specific for Thy-1.1 and Thy-1.2 determinants have been produced [17,18] and we used the MRC OX-7 mouse monoclonal antibody which has an affinity of 3×10^8 M^{-1} for binding to the

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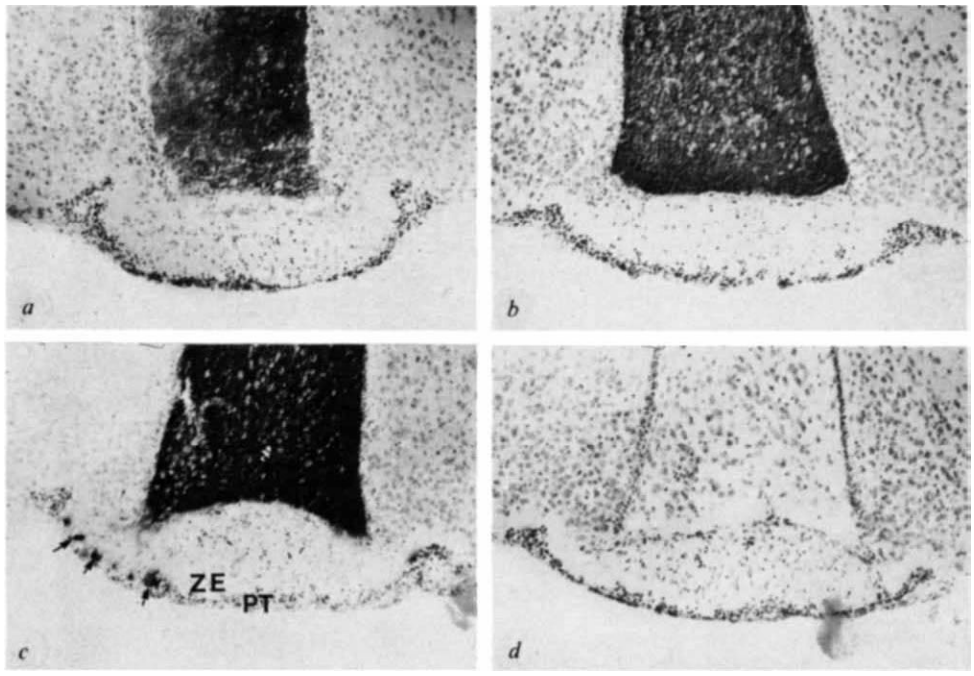

Fig. 2 *a*, Direct immunoperoxidase staining for Thy-1.1 antigen clearly shows the graft tissue 14 days after transplantation. There is no evidence of outgrowth of fibres from the graft. *b*, In this section taken from a 30-day transplant animal there is again no evidence for fibre outgrowth. *c*, 60 days after grafting, Thy-1.1-positive material (arrows) can be seen in the zona externa (ZE) of the median eminence in close contact with the pars tuberalis (PT). It is in this region that the pituitary portal vessels are found. *d*, Control for direct immunoperoxidase staining in the same animal as *c*. The total lack of staining indicates that the tissue arrowed in *c* must have emanated from the graft. ×420.
Methods: The animals used as recipients in these studies were 60–90-day-old *hpg* female mice reared in the Department of Human Anatomy, Oxford. The original mutants arose in backcrosses of the F_1 hybrids between two inbred strains C_3H/HeH and 101/H, and the present stock is maintained against this mixed background. Donor tissue was taken from 16–18-day-old fetuses removed from pregnant females of the AKR strain (Olac). The POA was dissected out as described in ref. 6 and placed in a drop of ice-cold saline. POA tissue from two donors was taken up with a minimal volume of saline into a modified 22-gauge hypodermic needle with a stainless-steel plunger. The recipient mice were anaesthetized with ether and held in a rat stereotaxic machine with modified ear bars. The incisor bar was fixed 5 mm above the interaural line and the needle plus grafts was placed 3.5 mm anterior to the interaural line in the midline and lowered 5.5 mm down from the dura. The graft tissue was expressed from the carrier by depressing the plunger.

Thy-1.1 determinant[17] and does not react with the Thy-1.2 determinant.

We studied the use of Thy-1 allotypes as markers for tissue grafted into *hpg* mutant mice (see Fig. 2 legend for origin of *hpg* mice). In these mice the gonads do not develop postnatally due to a failure of production of the hypothalamic gonadotropic hormone-releasing hormone (GnRH)[19]. This peptide is produced by neurones in the preoptic area (POA), and grafts of normal 17–18-day old fetal POA in the third ventricle of mutant mice elicited the synthesis and release of pituitary gonadotropic hormones and subsequent testicular development[6].

Brain tissue from *hpg* mice did not react with MRC OX-7 antibody and these mice are presumably of the Thy-1.2 allotype. Accordingly, POA grafts from fetal AKR mice (Thy-1.1) were implanted into female *hpg* mice. Physiological function of the grafts was assessed by vaginal opening and increased uterine weight. In successful transplants, vaginal opening first occurred at about day 14, with cornification occurring at about day 17. Uterine weights of animals killed at days 14, 20–30 and 60 days after grafting were 4-, 9- and 12-fold higher than in untreated *hpg* females of the same age.

To detect the grafted tissue by labelling of the Thy-1.1 antigenic determinant, cryostat sections were cut at days 14, 20–30 and 60 after grafting and AKR fetal sections were also examined. The sections were first stained indirectly using unlabelled MRC OX-7 antibody followed by peroxidase-conjugated rabbit anti-mouse IgG antibody. The grafted tissue was clearly seen in the third ventricle (compare Fig. 1*a* and *b*), but staining of other material which was thought to be mouse IgG in blood vessels was also seen (Fig. 1*a*, *b*). To eliminate this background staining, peroxidase was directly conjugated with $F(ab')_2$ of MRC OX-7 antibody and this gave the results shown in Fig. 1*c*. No labelling of any sort was seen except for that of the grafted tissue. This labelling was completely blocked by the addition of pure rat Thy-1 antigen (which carries the Thy-1.1 determinant) to the antibody (Fig. 1*d*).

Using these labelling methods, some points of physiological interest were established. Sections of 16–18-day AKR fetal brain showed virtually no labelling with anti-Thy-1.1 antibody (not shown), yet the grafts showed easily detectable Thy-1.1 antigen by day 14 (Fig. 2*a*). This suggests that the fetal tissue has developed adult characteristics at least with respect to this marker. The grafted tissue had a physiological effect by day 14, yet no fibre outgrowth could be seen at this time in the region of the median eminence where the pituitary portal vessels are found (Fig. 2*a*). At 20–30 days there was still no evidence of any fibre outgrowth (Fig. 2*b*). By 60 days after grafting, fibres were seen approaching the portal vessels and concentrations of labelling occurred adjacent to these vessels (Figs 2*c*, 3*a*). This labelling was clearly specific, as shown by blocking with pure Thy-1 in Fig. 2*d*. In some cases, outgrowths of fibres were also seen at a site posterior to the median eminence (Fig. 3*b*).

Taken together, the results suggest that the initial phase of gonadal and therefore pituitary activation is elicited by GnRH released into the cerebrospinal fluid or into blood vessels supplying the graft. Subsequently, fibres grow out from the graft to reach the zona externa of the median eminence. In a previous study on male *hpg* mice[6], GnRH was detected in some outgrowing fibres by labelling with antibody but it is not known whether all the fibres as seen in Figs 2*c* and 3*a* are GnRH-positive. It is also not known if the fibres growing out at the posterior end of the graft (Fig. 3*b*) contain GnRH. The specificity of production of GnRH in outgrowing processes could be tested by labelling alternate serial sections for Thy-1.1 and GnRH.

The results in Figs 2 and 3 show the first applications to brain grafts of immunohistology based on the Thy-1 antigenic determinants. In the *hpg* mouse system, applications could be extended using an anti-Thy-1.2 antibody–peroxidase conjugate to test for growth of host tissue into grafts. More generally, the markers could be used to determine the fate of any neuronal grafts between Thy-1.1- and Thy-1.2-type mice and grafts made at the fetal level may be of particular interest. In grafting involving peripheral nerves, immunological rejection may be more of a problem than with CNS grafts, where close matching of donor and recipient strains is not essential, presumably because the CNS is an immunologically privileged site[20,21]. However, mouse strains which differ at the *Thy-1* locus but are very similar in the rest of their genome have been bred[22] and

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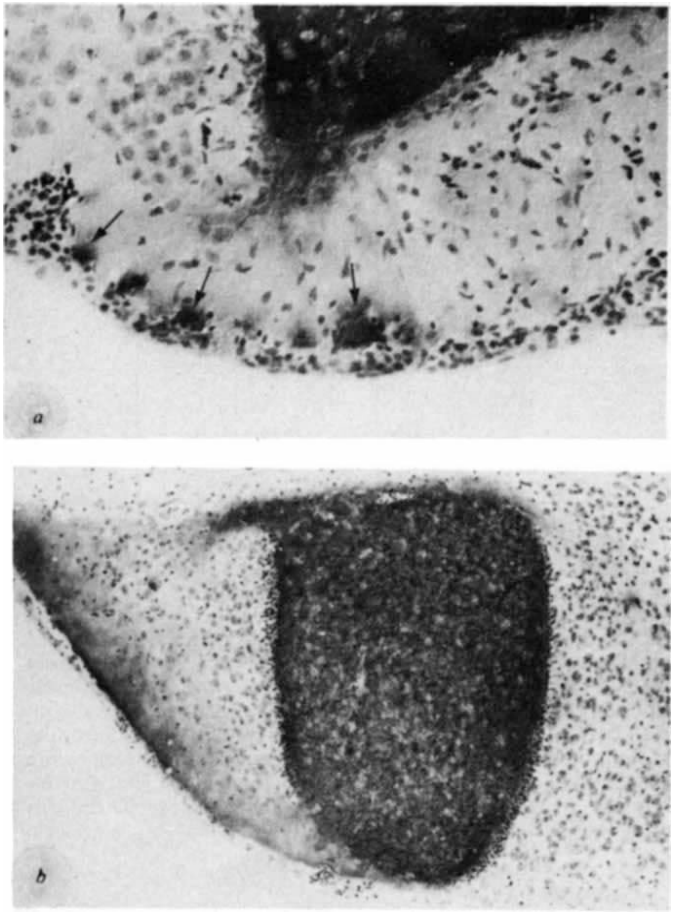

Fig. 3 *a*, Higher power (×1,000) magnification of the Thy-1.1-positive material (arrowed) in the region of the pars tuberalis/pituitary portal vessels 60 days post-transplantation. The use of Normarksi optics clearly gives the impression of fibres leaving the lateral edge of the graft and coursing towards the zona externa. *b*, This section, taken from the same transplant brain as in *a*, lies posterior to the median eminence and shows Thy-1.1 fibres leaving the graft both inferiorly and superiorly. (×600).

grafting of peripheral nerves may be possible between these strains.

Studies on grafting between mice and rats are also of interest, for it has recently been shown that mouse nervous tissue survives in rat brains, with mouse donor fibres being identified by their positive content of dopamine[23]. All rat strains so far studied have the Thy-1.1 determinant but are negative for Thy-1.2 and thus grafts between rats and Thy-1.2 mouse strains would allow distinction between donor and host tissue. This could also be done between any mouse strains and rats on the basis of other antigenic determinants of Thy-1 which are rat or mouse specific but which do not show polymorphism within the species[11].

We thank Alison Jones, Ed Davey and Don Etheridge for technical assistance and Rosa Beddington for helpful discussions. This work was supported in part by grants from the MRC and Wellcome Trust.

Received 29 June; accepted 19 August 1983.

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Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus

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In infected cells, the RNA genomes of RNA tumour viruses are copied into DNA by a virus-encoded reverse transcriptase enzyme[1–3]. This transfer of information from RNA into DNA was thought to be a unique feature of RNA tumour viruses[3], but recent results suggest it may be a more general strategy. Hepatitis B virus (HBV) has a double-stranded DNA genome, and it has recently been shown that the minus DNA strand of the HBV genome is copied from a plus-strand RNA template, leading to the suggestion that reverse transcription is central to the life cycle of HBV[4–6]. More recently it has been suggested that the replication cycle of a plant virus, cauliflower mosaic virus (CaMV), includes a reverse transcription step[7–9]. We report here the existence of amino acid sequence homology between retroviral reverse transcriptase and the putative polymerases of HBV and CaMV.

Homologies between distantly related proteins can be detected using the presently available data on the substitution frequencies between different pairs of amino acids[10–12] (when proteins evolve under the constraints of maintaining their original functions, conservative amino acid substitutions are much more frequent than radical ones[10–15]). Using this strategy, the amino acid sequences of reverse transcriptase (*pol*) gene products of Moloney murine leukaemia virus (M-MuLV)[16] and Rous sarcoma virus (RSV)[17] were compared. Two regions of apparent homology were found, at the N- and C-termini of the proteins, suggesting that these regions are functionally important (Fig. 1*a*), but on the whole the two genes seem to be only distantly related.

Similarly, we have compared the amino acid sequences of the M-MuLV and RSV *pol* gene products with those coded for by all the unassigned open reading frames of HBV (adr strain)[18] and CaMV (CM 1841 strain)[19]. The amino acid sequences coded for by the longest open frames (but not the other open frames) of the HBV (Fig. 1*b*) and CaMV (Fig. 1*c*) were found to be homologous to those of the N-terminal conserved region of retrovirus *pol* gene products. Although polymerase genes have not yet been assigned for both the HBV and the CaMV, the longest open frames are considered the most likely candidates to be the genes, on the basis of the predicted molecular weights of their encoded products[4,7]. The homologies reported here support these predictions, so here we tentatively refer to these open frames as putative polymerase genes.

Figure 2*a–c* show the alignments of sequences that were compared in Fig. 1*a–c*. These aligned sequences exhibit marked similarity: of the total positions compared, 56, 53 and 47% are occupied by residues that are identical or chemically similar for the comparisons shown in Fig. 2*a*, *b*, and *c*, respectively (gaps were considered as substitutions between dissimilar amino acids regardless of their length). Furthermore, statistical tests show

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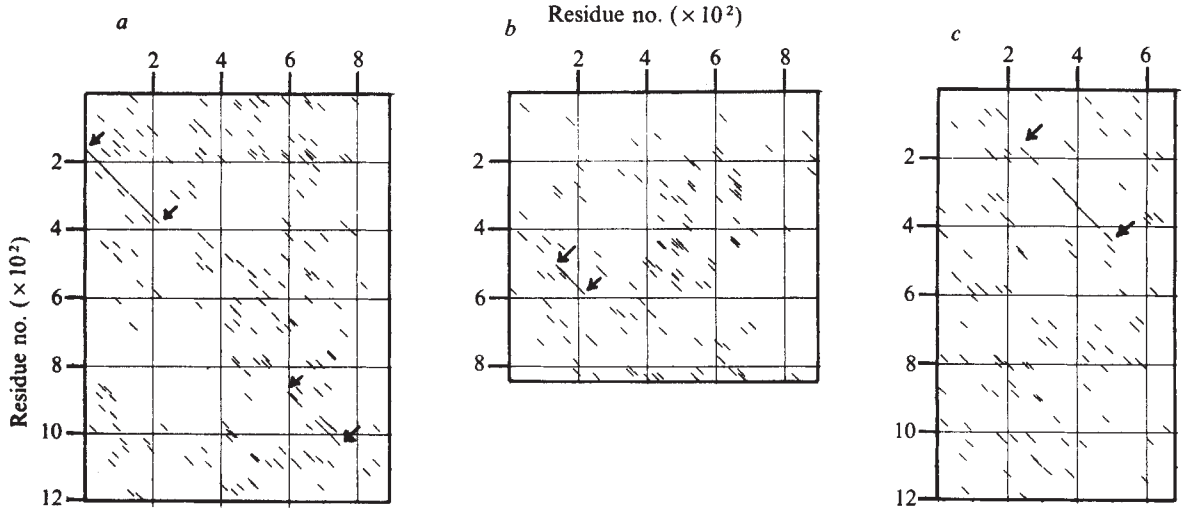

Fig. 1 Homology matrix comparisons of amino acid sequences encoded by polymerase genes of two retroviruses, and putative polymerase genes of HBV and CaMV. ***a***, M-MuLV (ordinate) against RSV (abscissa); ***b***, HBV (adr strain) against RSV; ***c***, M-MuLV against CaMV (CM 1841 strain). A computer program (H.H. and T.M., unpublished) was used to generate diagonal lines indicating segments of 20 residues long that show similarity above a threshold level. Arrows indicate regions where amino acid sequences were aligned (see Fig. 2). Sequence data were taken from refs 16, 17, 18 and 19 for M-MuLV, RSV, HBV (adr) and CaMV (CM 1841), respectively. **Methods:** we previously showed that the relative frequency of amino acid substitutions in protein evolution, decreases linearly with the increase in physicochemical (polarity and volume) difference d_{ij} between amino acids a_i and a_j for a range $0 \leq d_{ij} \leq 3.5$, while the frequency of substitutions between more dissimilar amino acids ($3.5 < d_{ij} < 5.1$) is relatively low and seems to be independent of d_{ij} (ref. 12). Thus, for a pair of amino acids a_i and a_j, a score s_{ij} was assigned as $s_{ij} = 100$ for $d_{ij} = 0$ (pair of identical amino acids), $s_{ij} = 100 \times (1 - d_{ij}/3.5)$ for $0 < d_{ij} \leq 3.5$, and $s_{ij} = 0$ for $d_{ij} > 3.5$ (for the values of d_{ij}, see ref. 12). Note that only 21 amino acid pairs (10%) out of all possible pairs fall within the range $d_{ij} > 3.5$. Average score values were calculated for pairs of segments of 20 residues long whose amino acid sequences were compared between different viral proteins and if the average score value is greater than 65, a diagonal line was generated on the corresponding position of the homology matrix. This threshold value is the degree of similarity that would occur by chance with a probability of less than 2×10^{-4}.

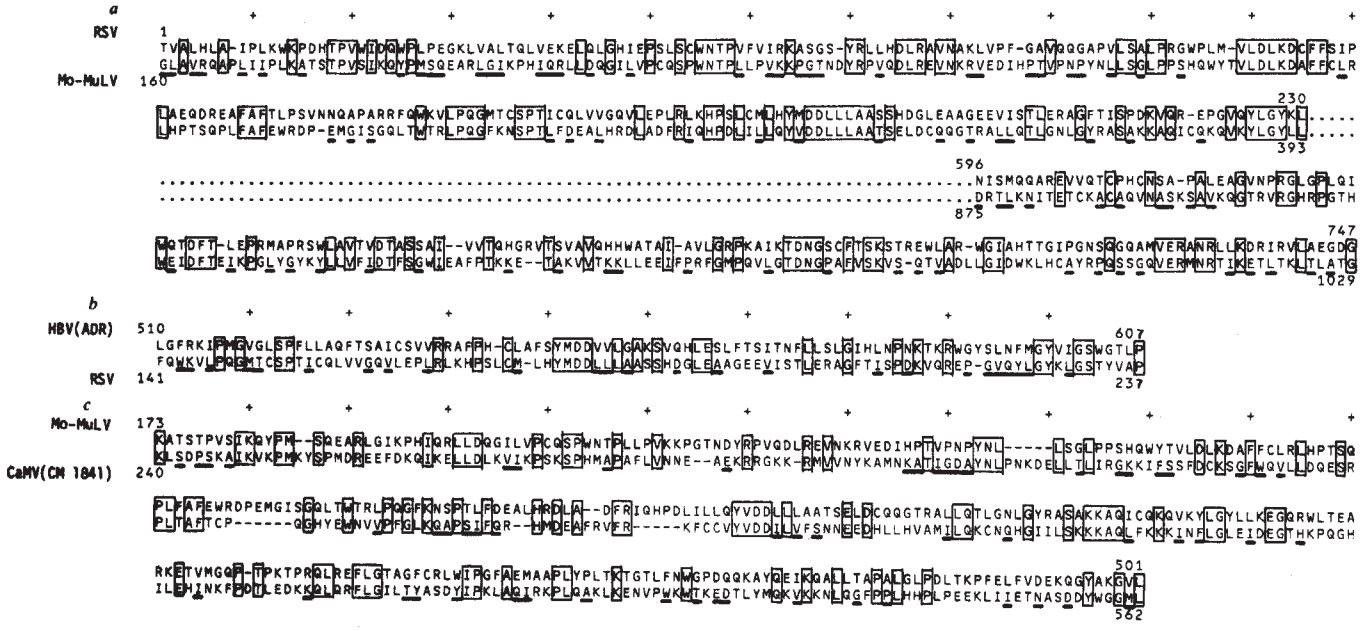

Fig. 2 Alignments of viral polymerase and putative polymerase gene products. On the basis of the homology matrices of Fig. 1, amino acid sequences of segments indicated by arrows in Fig. 1 were aligned for each of the viral pairs. Identical residues are boxed and favoured (conservative) amino acid substitutions are underlined. Favoured substitutions are defined as pairs of residues, both of which belong to the same group[10], the groups being as follows: A, S, T, P and G; N, D, E and Q; H, R and K; M, L, I and V; F, Y and W. Gaps (—) were inserted to increase similarity. A highly divergent region where no reasonable alignment is possible between RSV and M-MuLV (see Fig. 1*a*) is represented by dots. Statistical tests were made to assess the extent of similarity between aligned sequences[10,33]. Using the score matrix MDM_{78} (ref. 10), the score per residue(s) for the aligned sequences was calculated; gaps were counted as one substitution regardless of their length and a score of −60 was assigned for a gap[10]. Thirty pairs of randomized sequences having the same amino acid compositions as those of the real sequences were generated and the highest possible score per residue for each pair of randomized sequences was estimated by a computer algorithm[34]. The mean ($\bar{x}$) and standard deviation (s.d.) of the scores of 30 such pairs were calculated and the alignment score A of the real sequences, defined as $A = (s - \bar{x})/\text{s.d.}$, was then evaluated. The values obtained were as follows: for the N-terminal portion of *a*, $s = 16.28$, $\bar{x} = 2.70$, s.d. = 1.04 and $A = 13.08$ ($P < 10^{-9}$); for the C-terminal portion of *a*, $s = 8.55$, $\bar{x} = 0.96$, s.d. = 1.29 and $A = 5.87$ ($P = 2 \times 10^{-9}$); for *b*, $s = 20.23$, $\bar{x} = 1.28$, s.d. = 1.57 and $A = 5.72$ ($P = 5 \times 10^{-9}$); for *c*, $s = 9.79$, $\bar{x} = 2.45$, s.d. = 0.87 and $A = 8.47$ ($P < 10^{-9}$). These results indicate that the similarities between sequences compared are highly significant for all the cases. For references of sequence data, see Fig. 1.

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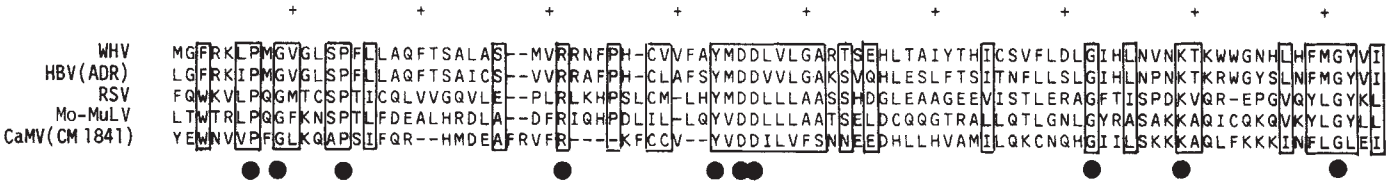

Fig. 3 Alignment of polymerase and putative polymerase gene products among five different viruses. WHV (Woodchuck hepatitis virus), residue positions 544–634; HBV (adr strain), 510–600; RSV, 141–230; M-MuLV, 303–393; CaMV (CM 1841 strain), 368–453. Gaps (—) were inserted to increase similarity. Boxed residues indicate identities or favoured amino acid substitutions (see Fig. 2) among four or five viruses. ●, Positions that are invariant among the five viruses. Sequence data were taken from refs 16, 17, 18, 19 and 35 for M-MuLV, RSV, HBV, CaMV and WHV, respectively.

that the similarities between the aligned sequences are highly significant for all the cases (the probability that such similarity is realized by chance is less than 10^{-8}, see Fig. 2).

In Fig. 3 the amino acid sequences of the products of polymerase and putative polymerase genes of five different viruses are aligned. Ten residues out of 94 can be seen to be invariant, and the middle region of the alignment appears to be particularly strongly conserved. It is quite striking that homology is observed between such widely different types of viruses.

That sequence homology has been observed between such different types of viruses suggests that it really is of functional and evolutionary significance. Analysis of the nucleotide sequences of closely related strains of HBV, of a similar type to that described previously[20–22], suggests that the strong amino acid sequence conservation in the homology region is, in fact, the results of functional constraints on the encoded product (data not shown). Further sequence comparisons revealed no homologies between these polymerases, and putative polymerases, and other polymerases of animal viruses and *Escherichia coli* (sequences from refs 23–28), suggesting that the homology region is involved in a function unique to reverse transcriptases.

The *pol* gene of retroviruses codes for at least three enzymes with different activities in a single transcription unit: DNA polymerase, RNase H and DNA endonuclease[2,29]. The DNA endonuclease activity appears to reside in the C-terminal region of the *pol* gene product[29,30], and we have found no homologies between the highly conserved region reported here and RNase H of *E. coli*[31], so it is a distinct possibility that the homology region is involved in the DNA polymerase activity in some way.

After submission of this letter, the complete nucleotide sequence of human adult T-cell leukaemia virus (ATLV) was reported[32]. Comparisons of the amino acid sequence of the putative *pol* gene product of this human retrovirus with those of M-MuLV and RSV revealed similar patterns of homology to those shown in Fig. 1*a*, indicating that these viruses are evolutionarily related to each other. The amino acid sequence of a region (positions 148–238)[32] of the ATLV is remarkably similar to the aligned sequences in Fig. 3; in this region, the sequence of ATLV can be aligned with that of M-MuLV without gaps and 10 invariant positions of Fig. 3 are totally conserved even when the ATLV sequence is included.

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

Received 27 June; accepted 23 August 1983.

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An immobile nucleic acid junction constructed from oligonucleotides

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Base-paired DNA duplexes involving oligonucleotide model systems have provided the major source of detailed structural and dynamic information about double helical structure[1]. Triple- and quadruple-branched 'junction' structures of DNA have a transient existence as intermediates in the replication or recombination of DNA molecules[2–5] while cruciforms may be inducible by negatively supercoiling closed circular DNA[6–11]. However, it has not been possible to investigate these forms structurally at high resolution in short-chain molecules, where the junction will yield a significant component of the signal, because these naturally occurring intermediates are inherently unstable, due to internal sequence symmetry, which permits their resolution to double helices, via branchpoint migration[12–15]. We have recently proposed that migration can be eliminated to yield immobile junctions from oligonucleotides[16–19] by combining sequence symmetry constraints with equilibrium calculations. We present here electrophoretic and UV optical absorbance experiments which indicate that four hexadecadeoxynucleotides (Fig. 1) indeed do form a stable tetrameric junction complex in solution.

The electrophoretic mobility of a nucleic acid oligomer in non-denaturing conditions is a function of its size, shape and extent of base pairing[20,21]. When the individual strands in Fig. 1, or equimolar mixtures of pairs, triplets and the tetrad corresponding to the complete junction, are subjected to electrophoresis, the patterns shown in Fig. 2 result: lanes *c–f* show

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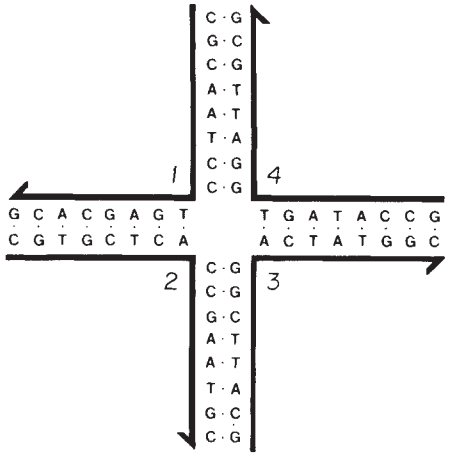

Fig. 1 An immobile nucleic acid junction composed of four hexadecanucleotides. This sequence has been designed by using the sequence symmetry minimization rules, supplemented by equilibrium distribution optimization[16–19]. The strand numbering indicated is used throughout the text. Note the lack of 2-fold symmetry around the centre, so that migration is not possible. This sequence also contains no repeating GpG sequence longer than two, in order to minimize this form of non-Watson–Crick pairing also. These sequences were commercially synthesized by phosphotriester techniques.

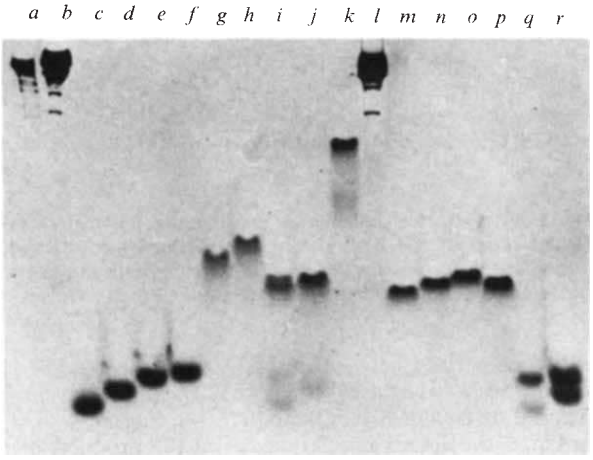

Fig. 2 Polyacrylamide gel electrophoresis of oligodeoxynucleotide strands and mixtures. Each well contained 2.5 μg of each strand, alone or in combination with others. Lanes *a*, *b* and *l* contain restriction digests of ΦX174RFDNA; *a* is the *Hin*fI digest, *b* the *Hae*III digest and *l*, is the *Hin*cII digest. The lowest molecular weight fragments in these digests are: 42, 48, 66 and 82 (*Hin*fI), 72 and 118 (*Hae*III) and 79 and 162 (*Hin*cII). Lanes *c–f* contain strands 1, 2, 3 and 4 respectively. Lane *g* contains an equimolar mixture (based on extinction coefficients derived from dry weights) of strands 1, 2 and 3; lane *h*, 1, 2 and 4; lane *i*, 1, 3 and 4; lane *j*, 2, 3 and 4. Lane *k* contains an equimolar mix of strands 1, 2, 3 and 4. Lanes *m–r* contain equimolar mixtures of pairs of strands: *m* contains 1 and 2; *n*, 3 and 4; *o*, 1 and 4; *p*, 2 and 3; *q*, 1 and 3; and *r*, 2 and 4.

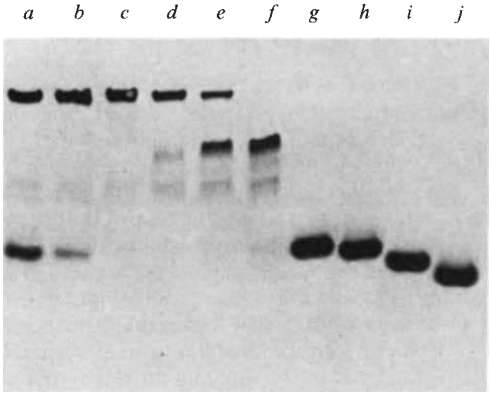

Fig. 3 Stoichiometry of mixing in the four-component complex. In this experiment, electrophoresis of mixtures containing different ratios of two components was carried out: component (i) consists of an equimolar mixture of strands 1, 2 and 4, while component (ii) consists of strand 3 alone. Lanes *g–j* contain 8 μg of free strands 4, 3, 2 and 1, respectively. Lane *f* contains 6 μg of component (i). Lanes *a–e* each contain 6 μg of component (i), plus component (ii) in the following amounts (μg): *a*, 4; *b*, 3; *c*, 2; *d*, 1; and *e*, 0.5.

the mobility of the individual strands. Figure 2, lanes *m–r* contain each of the six possible pairs of strands in equimolar mixtures. The first four of these binary mixtures are combinations which should form an arm of the junction. The last two mixtures do not correspond to an arm, but rather represent the diagonal combinations which should not associate. The mixtures which correspond to an arm of the junction (Fig. 2 *m–p*) migrate as single bands, with mobilities markedly less than those of any of the single strands. The two diagonal mixtures in lanes *q* and *r* migrate as single strands, with mobilities that correspond to their components.

Figure 2 *g–j* contains the four possible equimolar triplet complexes which can be formed by omitting each of the four strands from the tetrad in turn. The major component of each of these lanes is again a single band, travelling slower than the paired bands. Lane *k* contains an equimolar mixture of all four strands. This mixture travels as a single band, with a mobility lower than any of the other oligomeric mixtures. The presence of a single band with appreciable mobility in the lane corresponding to the tetrameric complex indicates that a molecular species with a well defined stoichiometry predominates. Higher unclosed complexes (1 : 2 : 3 : 4 : 1 : 2 : 3 : 4 : 1 . . .) thus cannot represent a significant fraction of the total material present. This finding is in accord with earlier predictions[18,19].

The stoichiometry of the strands in the complex involving all four strands can be estimated from the gel electrophoresis experiment shown in Fig. 3. In this experiment, an equimolar mixture of strands 1, 2 and 4 [component (i)] was titrated with strand 3 [component (ii)]. Figure 3*f* shows the mobility of component (i) alone, while lane *h* contains only component (ii). Figure 3*c* shows that mixing components (i) and (ii) in equimolar ratios leads to a complex with a single major component. No excess free single strands of (ii) occur when (i) is in excess (Fig. 3*d*,*e*). By the same token, free single strands do accumulate when component (ii) is in one-half molar excess, as seen in Fig. 3*b*. In conjunction with ^{1}H-NMR data (to be published elsewhere) indicating that strands 3 and 4 form a 1 : 1 complex, we conclude that the stoichiometry of the junction formed is indeed 1 : 1 : 1 : 1.

The slope of the Ferguson plot of electrophoretic mobility versus acrylamide concentration is a means of estimating the frictional constant of any molecular species[22]. In Fig. 4*a*, the complex is compared with 36- and 72-bp restriction fragments; from the slopes shown, the four-stranded structure has a friction constant quite different from linear duplex DNA of approximately the same size, or indeed of any size. Thus, the junction is a structure distinct from linear duplex DNA.

Next, the relative stability of the different complexes of strands 1 to 4 was assessed by thermal denaturation studies monitored by hyperchromism at 260 nm[23]. Figure 4*b* shows the thermal transition profiles of the individual strand 3 and an equimolar mixture of strands 1 and 2, compared with that of the quaternary complex at half the total strand concentration. The fact that the hyperchromism in the junction is twice as great as in the same concentration of pairs strongly suggests that the complex is closed, with four arms nearly intact. The increase in junction stability (seen in the higher melting temperature, T_m) further strengthens this argument.

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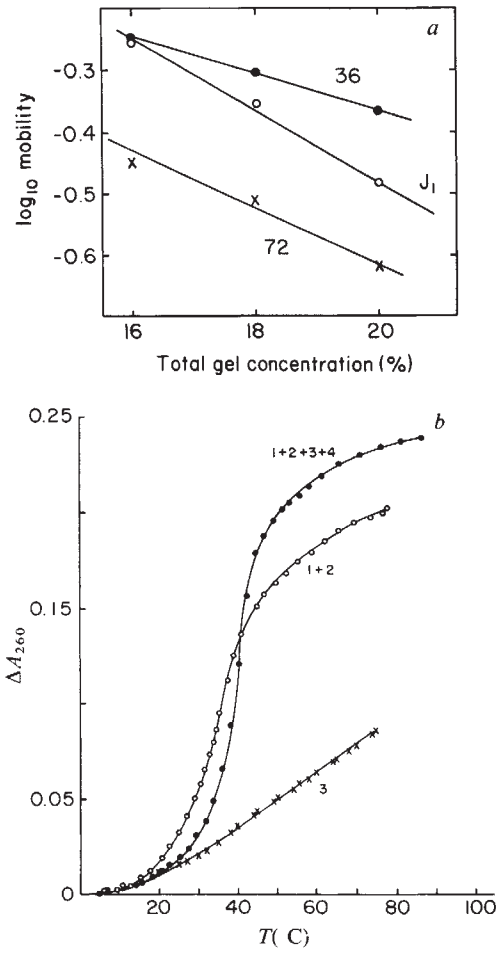

Fig. 4 Physical characterization of the junction. *a*, Ferguson plot of the equimolar complex of strands 1, 2, 3 and 4, indicated as J_1, compared with linear DNA duplexes containing 36 and 72 base pairs. Gels were cast with different compositions of total acrylamide, and the mobility of the four-component complex measured with respect to that of a reference dye, xylene cyanol FF, and reference duplexes of 36 and 72 bp. The smaller reference is a cloned fragment of *Escherichia coli lac* operator, the larger is the lowest molecular weight duplex from a *Hin*f digest of ΦX174 RF DNA. *b*, Thermal transition profiles of the quaternary complex (25 μM total strands), an equimolar mixture of strands 1+2 (49 μM total strands) and individual strand 3 (98 μM total strand) at 260 nm. The UV absorbance of the samples was measured at each of a series of temperatures (T), and the results expressed as $A_{260} = A_{260}(T)/A_{260}$ (10 °C) − 1. Typical A_{260} values for high-molecular weight DNA duplexes approach 30% (ref. 24); actual values depend on base composition, length and solvent. Fragment 3 alone exhibits a typical non-cooperative transition characteristic of nucleic acids in the absence of base pairing[24]. Note that the concentration of 4-fold complex is 6.25 μM, while that of the paired arm is 24.5 μM. Hence, the hyperchromicity in the junction is actually more than four times that of a single arm.

The present level of characterization does not allow us to conclude that the junction is completely immobile, despite the presence of non-complementary sequences flanking the junction. Further experimentation is needed to establish that a limited amount of non-Watson–Crick mobility is not occurring in the vicinity of the junction.

The above experiments clearly show that it is possible to design and synthesize immobile nucleic acid junctions by using optimization procedures[16–19]. We have a second synthetic junction, composed of dodecameric fragments (N.R.K., R.-I.M., A. J. Wand, G. H. Veeneman, J. H. van Boom and N.C.S., in preparation), which we have compared with the hexadecameric junction reported here. Gel electrophoresis analysis indicates that while this material also forms a junction, alternative pairings may occur unless optimization statistics are as favourable as those of the hexadecamers discussed here[18,19].

This work was supported by NIH grants GM-29554, ES-00117 and CA-31027. N.C.S. is the recipient of a NIH Research Career Development Award. We thank Dr P. Lu for his 36-bp restriction fragment.

Received 6 June; accepted 11 August 1983.

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Sensitivity of circular dichroism to protein tertiary structure class

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Circular dichroism (CD) spectra of proteins are particularly sensitive to protein secondary structure[1–7]. Proteins have been divided into four classes on the basis of their secondary structures[8]; all-α (predominantly α-helical), all-β (predominantly β-sheet), α+β (separate α-helix- and β-sheet-rich regions), and α/β (intermixed segments of α-helix and β-sheet). In principle, CD should be able to distinguish between all-α, all-β and α-β (α+β and α/β) proteins. We show here that, in fact, CD can distinguish between all four classes of protein, and that the CD spectra of members of each class have certain common features.

All-α proteins exhibit a characteristic α-helix CD spectrum, as exemplified by that of myoglobin (Fig. 1*a*). CD spectra for myoglobin[7], haemoglobin[7], parvalbumin[9], cytochrome b_{562} (ref. 10), cytochrome *c* (ref. 5), haemerythrin (unpublished data) and polyglutamic[7] acid all have two distinct negative minima at about 208 and 222 nm, a positive peak in the 190–195 nm region, and a cross-over from positive to negative below 172 nm. These three characteristics are sufficient to separate the all-α proteins from the other types. Other characteristics, such as a shoulder around 185 nm and the high intensity of the CD spectrum that is indicative of large amounts of α-helix, are not essential features. Cytochrome *c*, which contains about 40% α-helix, has low intensity CD bands but meets all three essential criteria to be included in the all-α category.

The α+β and α/β classes have CD spectra that are dominated by the α-helix portion (Fig. 1*b*, *c*). Both classes exhibit the two negative bands around 208 and 222 nm and a positive

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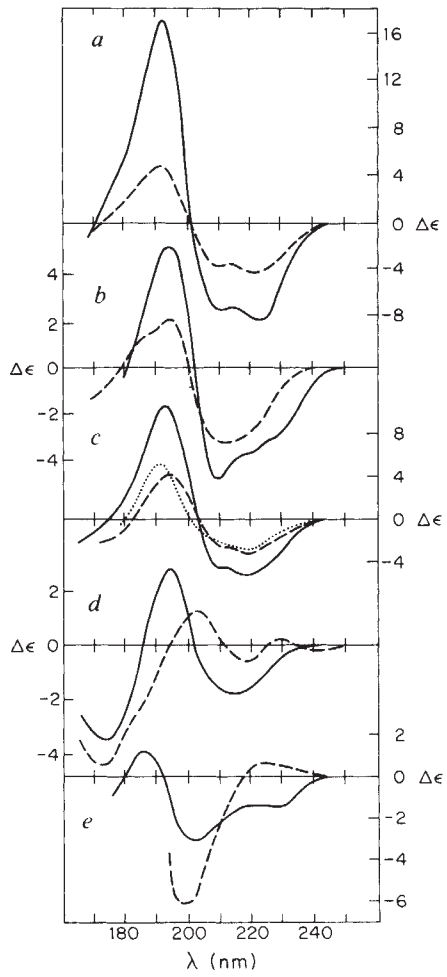

Fig. 1 Examples representing the extremes in CD spectra for the tertiary structural classes. *a*, All-α proteins: myoglobin (——) and cytochrome *c* (– – –); *b*, $\alpha+\beta$ proteins: lysozyme (——) and ribonuclease A (– – –); *c*, α/β proteins: triose phosphate isomerase (———), flavodoxin (– – –) and subtilisin BPN′ (· · · ·); *d*, all-β proteins: prealbumin (——) and Bence-Jones protein (– – –); *e*, all-β proteins: α-chymotrypsin (——) and soybean trypsin inhibitor (– – –). The spectra for myoglobin, lysozyme, ribonuclease A, subtilisin BPN′ and α-chymotrypsin were redrawn from Hennessey and Johnson[7] and soybean trypsin inhibitor from Mori and Jirgensons[24]. The other proteins were redrawn from Brahms and Brahms[5].

band in the 190–195 nm region. However, the cross-over from positive to negative is above 172 nm, which serves to differentiate the α-β proteins from the all-α type. Subtle differences in the CD serve to separate the two classes.

CD spectra of lysozyme[7], ribonuclease A[7], papain[7], insulin[11], cytochrome *c* peroxidase[12], thermolysin (unpublished data), and *Staphylococcus* nuclease[13] were analysed to determine the characteristics of the $\alpha+\beta$ type. In all cases the 208 nm band is of larger magnitude and more prominent than the 222 nm band (Fig. 1*b*), and in some cases only a very shallow minimum is observed around 220 nm.

For the α/β class of proteins the CD spectra of subtilisin BPN′ (ref. 7), lactate dehydrogenase[7], triose phosphate isomerase[5], subtilisin novo[5], glyceraldehyde-3-phosphate dehydrogenase[5], flavodoxin[5], pyruvate kinase[5], aldolase[14], hexokinase[15], glutathione reductase[16], thioredoxin[17], phosphoglycerate kinase[18] and dihydrofolate reductase[19] were analysed. Here the 220 nm band is more pronounced than for $\alpha+\beta$ proteins (Fig. 1*c*). Large overlap between the 208 and 220 nm bands produces a single broad minimum in some cases (Fig. 1*c*), but the minimum is always skewed towards the 220 nm region.

All-β proteins have spectra that are characterized by the absence of the features characteristic of α-helices (Fig. 1*d*, *e*). These spectra are low in intensity, and exhibit a variety of shapes. Apparently, the contribution to the CD of the β-turns and other structures are comparable with the contributions of the β-sheet.

α-Cobratoxin[20], Bence-Jones protein[5], gene 5 protein[21], immunoglobulin[22], prealbumin[5], Cu,Zn-superoxide dismutase[5] and concanavalin A[5] are all-β proteins that exhibit a minimum in the 210–220 nm range (Fig. 1*d*). They also have a second negative minimum in the 170–180 nm range, and a cross-over from positive to negative above 185 nm. The spectra of Bence-Jones protein and gene 5 protein bear a certain resemblance to the model β-sheet spectrum. In contrast, the CD spectra observed for a second set of all-β proteins resemble the spectrum of random coil models[5,23]. Soybean trypsin inhibitor[24], wheat germ agglutinin[25], rubredoxin[5], elastase[7] and α-chymotrypsin[7] have a minimum around 200 nm and a positive band in the 185–190 nm range (Fig. 1*e*). Crystal structure data of these proteins show that their antiparallel pleated sheets are either very much distorted or form very short irregular strands (see ref. 26 and references therein). This irregularity may cause the negative CD band to shift from the ideal β-sheet position (210–220 nm) towards the 200 nm region. The $\alpha+\beta$ proteins that have minima in the same region exhibit the distinctive shape associated with the α-helix, so the all-β proteins are easily distinguished.

The CD spectrum of a protein reflects the overall picture of the molecule. It depends on all the amide groups, not just those in the α-helix and β-sheet categories. Whilst we might expect exceptional protein structures to have exceptional CD spectra, nevertheless, we find correlations between CD and tertiary structural class that are highly reliable. For particularly large proteins that contain many domains, it would be more meaningful to investigate the tertiary structural class for each domain. This might be done by cleaving the proteins into major fragments[27] and subjecting each fragment to CD analysis. Prediction of secondary structure from amino acid sequence can now be combined with analysis of CD spectra for the fraction of secondary structure and the tertiary structural class to aid in elucidating the structure of proteins that have not been crystallized.

This work was supported by PHS grant GM-21479 from the Institute of General Medical Sciences. We thank Mr J. P. Hennessey Jr for measuring some of the CD spectra and for helpful discussions.

Received 8 June; accepted 19 August 1983.

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The Ruling Stones

Colin Burgess

Stonehenge Complete.
By Christopher Chippindale.
Thames and Hudson/Cornell University Press: 1983. Pp.300. £12.50, $29.50.

ANYONE who caught Christopher Chippindale's common-sense words on BBC Radio, amidst the extraordinary annual midsummer pantomime at Stonehenge, will approach this book already impressed. Nevertheless his title is provocative, and a disarming waiver comes only in his conclusion: "Even a book of this size has room for only a fraction of everything that exists to do with Stonehenge". Within his chosen limits, however, he does offer a record that is strikingly comprehensive.

First, what this book is and is not: it is essentially the story of Stonehenge since its first medieval notice. Its concern is thus matters antiquarian, and with attitudes to the monument past and present, as befits the author's declared interest in taste and archaeological ideas. Do not expect a prehistory of Stonehenge and its period, for even the site's development is merely outlined. Major questions such as engineering and social implications receive scant consideration, and there is not even a list of radiocarbon dates.

A different title would have forwarned the reader, for the book strikes an idiosyncratic balance between the bizarre and the fundamental. While one welcomes the space given to druids past and present (very well done), be prepared for Roman Polanski's difficulties in filming at Stonehenge receiving as much space as Kellaway's suggestion of glacial transport of the bluestones, though Polanski makes the index and Kellaway does not. Yet the problem of how and when the bluestones reached Salisbury Plain is one of the most intriguing in British prehistory

But this book should be judged not for what may be a publisher's title but for what it is: an excellent and exhaustive history of Stonehenge in recent centuries. Lavish illustrations range from the first medieval manuscript depictions, through all the classic views and many less familiar, to such off-beat canvasses as a motor cycle petrol tank. Since the story of Stonehenge is a microcosm of attitudes to the past, this is an important historiographical work. The lack of a list of illustrations and a bibliography is, therefore, surprising. Advice on further reading and lengthy footnotes are no substitute, though frustrating for those bent on quick demolition of this book's claim to completeness.

These deficiencies, perhaps not the author's, are offset by his skilful handling of such difficult problems as ancient astronomy, his wit, and his pleasing way with words. Time and again his assessments strike just the right note. He surveys megalithic science at an optimum moment, when the crash of the Hawkins-Thom bandwaggon has been accepted in most archaeological and scientific circles. Mr Chippendale is on hand to reassemble the pieces into a sensible afterview of prehistoric man's celestial interest and surveying abilities. On the other hand he chooses a bad time to summarize the archaeology of Stonehenge, with rumours of basic tenets crumbling, and a major conference scheduled for Salisbury this autumn. So this is a book for antiquaries, those with a love of historical and social minutiae, and for those interested in the history of ideas, especially about our past. □

The first photograph of Stonehenge, a calotype taken by R. Sedgfield in 1853, (From Stonehenge Complete*).*

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An approach to the deity

W. H. McCrea.

God and the New Physics.
Paul Davies.
Dent: 1983. Pp.252. £8.95, $16.50.

THE text of this book is about the whole of existence. The title says it is about God. The conclusion is that these may mean the same. Paul Davies calls his subject a "search for God" and claims that "science offers a surer path to God than religion". In the book's closing sentence he declares his "conviction that only by understanding the world in all its many aspects . . . we will come to understand ourselves and the meaning behind this universe, our home".

The title signifies that the book is also about physics. Physics is about experience — the experience that certain operations produce certain results, the same operations always producing the same results. Physicists have the further experience of being able to construct theoretical (mathematical) models that can be put in correspondence with physical observation, and that suggest other observations and predict their outcome. The essential feature is that physics is throughout concerned with experience.

Although he mentions 'new' physics, it is the models that are new, in relation to those of classical physics. They have indeed led to fresh observations, but the operations performed in making them could have been performed at any time with the same results (unless some observation were designed to observe a change). The 'new' models in general give better accord with observation than did 'classical' models. It is commonly also said that they give better understanding; but this is saying the same thing because the only meaning of understanding here is the ability to produce a model.

Davies discusses the great problems of being — creation, life, mind, soul and self, free will and determinism — in the context of the new physics, which he expounds as needed. Broadly speaking, his procedure is

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Konrad Lorenz

The Foundations of Ethology

Translated from the German by the author and Robert W. Kickert

1981. 34 figures. XVII, 380 pages.
Cloth DM 48,–, approx. £ 12.00
ISBN 3-211-81623-2

"More than anyone else Konrad Lorenz is the intellectual architect of ethology. . . . His aim is to introduce modern workers to the often ignored indispensable core of ethological knowledge and how it was obtained. . . . This volume needs to be read and reread." *Science*

". . . a penetrating and sometimes acerbic criticism . . . Nobel Prize winner Lorenz is as sharp as ever, and students of ethology will find a gold mine in this book." *Los Angeles Times*

Genetics of Influenza Viruses

Edited by **Peter Palese** and **David W. Kingsbury**

1983. 62 partly coloured figures. XVI, 360 pages.
Cloth DM 128,–, approx. £ 32.00
ISBN 3-211-81743-3

This multi-authored book reviews the biochemical structure, the mechanisms of replication and the genetics of influenza viruses. These viruses have been associated with respiratory illness in man and animals as well as with Reye's syndrome in children. Recent studies using a variety of methods including genetic engineering techniques have contributed to a much better understanding of the replication steps of the virus, of its epidemiology and of the factors contributing to its pathogenic potential. Special emphasis is given to a discussion of the molecular basis for the variation of influenza viruses, which leads to recurrent epidemics and pandemics. The book also reviews the molecular basis for approaches to develop new influenza virus vaccines and anti-influenza virus agents.

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to conclude the treatment of each main topic with an enquiry as to what it may have to say about God in requiring or not requiring, or in qualifying, the concept. Some of the arguments are age-old, and Davies is learned in these. But he is more concerned with those related to the new physics and the 'understanding' of the world it is claimed to furnish. The trouble about this is that the understanding is tied to models. Models change. As physics goes on its way, the predictions of models interpreted in terms of possible observations seem in some sense to converge. On the other hand the models themselves diverge in character. Among some mentioned by Davies, we have only to think of those of Newton, Maxwell, Einstein, Bohr, Heisenberg, Dirac, Everett on to those of modern field theories, particle physics and cosmology, and the enormous differences between them.

The simple consequence is that no acceptable concept of God can be closely linked with current scientific theory. At any stage we do need some theory to provide a language with which to describe experience — much as we need a frame of reference in order to describe, say, a vector — but the experience is the enduring feature. So if God is to be found through science this must surely be by establishing closer links with experience rather than with theory. The experiential aspect of the search is religion. Thus the paths of science and religion may not be so greatly different. We still have to discover if they lead to what we should wish to call God.

The new physics does include features that do not depend on any particular model and that, once admitted, may never be discarded. One is the need to recognize the effect of the observer upon the outcome of an observation. This can lead for instance to the consideration of the devastatingly revolutionary interpretation of quantum theory proposed by H. Everett, as recalled by Davies. At any rate, realization of this seems to ensure that a God discovered by science cannot be considered separate from ourselves.

A concept to which Davies gives recurrent attention is that known to philosophy as 'holism', in contrast to 'reductionism', which is supposed to describe a more usual attitude in science. He infers, "Life is a holistic phenomenon", and proceeds to similar inferences about mind, soul, self, etc. It does not matter if this is partly a question of semantics; the first point that does matter is that we are able to recognize the existence of life, mind, etc. as part and parcel of the same universe that the physicists study. The relevance of the 'new' physics in this context is that the world of quarks, vacuum fluctuations, uncertainty principle, and so forth seems so readily to accommodate this existence. In fact it may seem more astonishing for the insubstantial world of modern fundamental physics to accommodate something so material as a high-rise block or a supertanker than the minds that designed them.

The second point is that, as Davies indicates, holistic phenomena evidently constitute a hierarchy. If we accept this we can scarcely then accept that the human mind or spirit is its pinnacle. The utmost holistic phenomenon is surely the universe itself. If so, it must subsume all others, mind, spirit, personality and the rest. May we call this God?

"In this context, God is the supreme holistic concept, perhaps many levels of description above the human mind". This is what Davies writes towards the end of the book. His meaning is a shade different from my description. And it is not evident whether Davies regards this as the culmination of his search so far as the book is concerned. For he proceeds to ask, in effect, what happens if the universe comes to an end? This seems to be a meaningless question; no-one could ever validly proclaim the end of the universe; if he could, he could not be there to do it. More generally, we must evidently recognize that there comes a natural limitation to our enquiries when either we do not know what question to ask, or we fall into an infinite regress of questions why.

This conclusion that God is all in all, and that we can understand Him only in terms of Himself, must be man's oldest idea of God. It owes nothing to any particular science. It seems to offer small consolation to the many like Davies who, as he has said elsewhere, share the belief that there is 'something behind it all'. The implication is that they believe there to be some element that is capable of making their activities more immediately meaningful and purposeful to themselves. Davies book and the contents of its 'select bibliography' demonstrate how widespread and insistent is this belief. So maybe the 'surer path' has by-passed something vital.

Occasionally a reviewer may offer a better tribute to an author by expressing thoughts stimulated by a book instead of attempting any close critical assessment of the work. In due humility when faced with Paul Davies' learning and courageous thinking (which he must have been long accumulating for this undertaking) and with his remarkable literary skills, I have for once ventured upon such a course. All I can hope is to have indicated the magnitude of the theme and something of Davies' approach to it, and above all to incite all to read this book. □

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Nature's Autumn Books Supplement will appear on November 10.

Among the contributors will be Peter Medawar, Eric Ashby and Isaac Asimov.

Books to be reviewed include *The Modularity of Mind* by Jerry A. Fodor, *The Return of Cosmology* by Stephen Toulmin and *Great Geological Controversies* by A. Hallam.

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Pathway to power

Stanley Salmons

Muscle Development: Molecular and Cellular Control.
Edited by Mark L. Pearson and Henry F. Epstein.
Cold Spring Harbor Lab: 1982. Pp.580. $66.

MOST cells are equipped with a primitive contractile system that enables them to move, change shape, divide, transport organelles, and so on. Skeletal muscle develops from cells such as these — myoblasts — in which the contractile proteins do not appear to differ from those of fibroblasts or amoebae; however, after differentiation it consists of multinucleate fibres in which contractility has developed to such a pitch that the tissue can perform work for the whole organism. In the process the myoblasts proliferate and fuse to form myotubes, which synthesize increasing amounts of proteins specific not only to muscle but also to the developmental stage of the muscle.

The study of muscle development therefore tends to focus on: the structure of muscle proteins and the way they vary in ontogeny and phylogeny; the structure and regulation of the corresponding genes in normal and mutant forms; the assembly of the proteins into cytoskeletal structures; membrane phenomena, including cell interactions and formation of neuromuscular junctions; and factors influencing all these processes during the course of development. These topics appear to have been well aired at the Cold Spring Harbor meeting which generated the present book.

Several contractile proteins have now been sequenced. Although traditional methods of analysis could not have been expected to succeed as quickly with the much larger myosin heavy chain, progress has in fact been remarkable because of the advent of the complementary technique of nucleotide sequence analysis of cloned genes. Moreover, DNA hybridization has shown that myosin heavy chains and actin from a variety of sources are encoded by multiple genes. Individual genes in these families have sequences showing considerable homology, but they also have specific sequences, and it is these that are responsible for the different isoforms.

The significance of all these isoforms is far from obvious, as many are known to be functionally similar; for example, the demonstrably different myosins of embryonic and adult chick pectoralis muscle are now shown to have similar enzymic activity (Lowey *et al.*). One attractive proposal (Fyrberg *et al.*) is that different gene copies may be coupled with different regulatory sequences, which ensure that they are expressed only at particular stages of development. An extension of this idea is that there are sets of genes, coding for diverse muscle proteins but under coordinate regulation. Until now this has been studied mainly in relation to myoblast differentiation (Hastings and Emerson) but the techniques should be transferable to later stages of muscle development. It will be possible to test such hypotheses as further information becomes available about the primary structure of the genes and of the DNA sequences flanking them (see for example Ordahl *et al.*).

However, there is already evidence for at least one alternative to transcriptional regulation of gene expression during muscle development: the unexpected finding that chicken actin genes undergo a transient amplification during myogenesis (Schwartz and Zimmer). This raises the whole question as to whether some of the polymorphism of muscle proteins during development may be the result of primary sequence rearrangements, of the type already found in the immunoglobin genes.

Muscle Development is not comprehensive in its coverage. It nevertheless provides a very useful overview of the state of progress in some of the most rapidly moving areas. The authors have, it seems, been encouraged to place their contributions in the context of what is known and what needs to be known. This, together with introductions prefacing each section, a concluding perspective by the editors, and an excellent subject index, will help to widen the book's appeal to a less specialized readership. □

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Spanning defence

J.H. Humphrey

Annual Review of Imunology, Vol.1.
Edited by William E. Paul.
Annual Reviews Inc: 1983. Pp.670. $27.

ONLY the improbable contingency that immunology ceased to develop and expand could have prevented the appearance sooner or later of *Annual Review of Immunology*. Chapters on immunological topics have been appearing in other long established journals (*Ann. Rev. Microbiol* and *Biochem. and Physiol.*) over a good many years. It says much for the effectiveness of *Advances in Immunology* (now at vol 34) and *Immunological Reviews* (now at vol 73) as well as *Progress in Allergy* and various immunologically oriented Current Topics series that *Ann. Rev. Immunol.* comes as 25th in the Annual Review family and that its birth has been delayed until 1983. Yet it is not unreasonable to ask what useful contribution may this new series be expected to make.

When *Annual Review of Biochemistry* began in 1932 it was readable for pleasure, even from cover to cover — and vols 1-3 were so read by this reviewer when a student! — because their chapters were surveys of particular topics by individual authors who had sufficient critical comprehension of the (admittedly limited) current knowledge of these topics to pull it together and make what seemed sense out of it. This ideal form of review becomes increasingly difficult to achieve as topics expand and become frayed at the edges, and when the experimental data are apparently conflicting or cannot be placed in a clear conceptual context. It could be argued that topics at such a stage are not ripe for review. But since the scientific world is full of people with practical problems, research students, and others, who wish to be kept up to date with the increasing and unassimilable volume of published work, the review does not always wait until the topic is ripe. Such reviews tend to be long and disjointed and therefore not easy to read.

A new review volume obviously cannot be expected to cover entirely fresh ground, but it would be justified and welcome if it selected topics which are ripe and reviewers who, without discarding their critical faculty, present a stimulating and/or elegant connected story rather than a compendium. This seems to be what the editors of *Ann. Rev. Immunol.* have aimed for and, by and large they have achieved it. The 21 review chapters average about 30 pages in length, and are generally built quite clearly around those aspects and hypotheses which their distinguished authors regard as important and to which they themselves have made substantial contributions. The range is wide. T-cells and their functions and regulation rate seven chapters; immunoglobulins and their genes three; soluble factors, idiotypes and epitopes and the major hisotcompatibility complex two each, and inflammation, complement, malaria, tolerance, autoimmunity and transplantation one each.

A novel and welcome feature is a highly personal historical and semi-philosophical opening chapter by Elvin Kabat, about what it was like getting started 50 years ago. In the space allotted to this review it is not possible to discuss the chapters in detail. However Volume 1, in my opinion, can be read from cover to cover, certainly for enlightenment and almost all for pleasure. If the standard can be maintained this series will be a real asset, and libraries will have to take it without question. □

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Putting song to use

John Krebs

Acoustic Communication in Birds. Vol.1. Production, Perception and Design Features of Sounds. Vol.2. Song Learning and Its Consequences.
Edited by Donald K. Kroodsma, Edward H. Miller and Henri Ouellet.
Academic: 1983. Vol.1. Pp.368. Vol.2. Pp.387. Vol.1. $36, £23.80; Vol.2. $39, £25.80.

NAOMI Mitchison once illustrated the liberated biological atmosphere of her family background by referring to early encounters with scientists whose lives were devoted to the study of subjects "as irrelevant as bird song". An alternative view comes from R.A. Hinde's introduction to these two volumes: "It is probably true to say that the study of bird song has done as much for the advancement of ethology as the study of any other specific aspect of behaviour".

The many ramifications and wide-ranging influence of bird song research is well illustrated in this collection of 17 original articles. Areas in which song-researchers are contributing important work include neuroanatomy and neuroendocrinology, genetic differentiation of natural populations, sensory perception, sexual selection, and learning. As impressive as the diversity of song research, is the way which common themes link together different approaches. For example, the "auditory template hypothesis" originally proposed by P. Marler (to whom the two volumes are appropriately dedicated) and his associates to account for results in studies of song development, is discussed not only in this context (by Kroodsma), but also in other chapters. It is referred to by Arnold as a starting point for neuroanatomical investigations of pathways of auditory feedback to 'song centres' in the brain and by Morton in a discussion of how it is that birds are able to assess the degree of degradation of sound as a result of its passage through the environment.

The role of song in mate choice is another recurrent theme — discussed in relation to maintenance of genetic isolation between populations at song dialect boundaries (Baker), in the context of the evolution of song complexity through sexual selection (Catchpole) and in accounting for the enlargement of the hyperstriatum ventrale which is associated with the acquisition of a large song repertoire in canaries and marsh wrens.

It is not only in bringing home these common themes that the book does a good job. Most of the individual chapters include thorough surveys with useful tables of literature on song learning, vocal mimicry, auditory sensitivity, song dialects, duetting and so on. It is hard to single out individual chapters for special praise since at least half are excellent. One general impression emerged: work on neural control, perception, motor production and physical aspects of transmission of bird sounds has made more progress than research on their functional significance — what they actually mean to both the birds that utter them and the birds that hear them.

Although various facets of vocal communication have been thoroughly documented for many years — vocal mimicry, song dialects, elaborate song repertoires of individual males, matched counter-singing between rivals, and so on — understanding their biological significance (or even understanding whether it is worth asking about their significance) seems to have progressed relatively little in the last decade or so. I am not sure why this is, but offer two suggestions. First, little successful thought seems to have been given to developing new techniques for answering questions about the biological significance of bird sounds; indeed much recent work still consists of description, correlation and interpretation with informed guesswork rather than incisive experiments. Second, perhaps the functional questions cannot be fully answered until more is known about the mechanisms of sound production and perception. To take a simple example, discussions of the significance of elaborate song repertoires are currently going on in the absence of any information abut how the birds themselves perceive complex sounds, whether they categorize and differentiate them in the same way as we do. Once there is a good base of information about categorical perception, it may be possible to view the question of functional significance in a new light.

Johannes Kepler wrote in his *Mysterium cosmographicum* "We do not ask for what purpose the birds do sing, for song is their pleasure since they were created for singing". While there has been progress since Kepler's day, there is still some way to go before we will know exactly why birds sing the way they do. □

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Modelling nuclei

P.E. Hodgson

Statistical Theory and Random Matrices.
By Moshe Carmeli.
Marcel Dekker: 1983. Pp.203. $35.

THE properties of individual low-energy nuclear states can be determined by nuclear reaction studies, and they can often be quite well understood using various nuclear models. At higher excitation energies the density of states becomes so large that it is impossible to study them individually. Instead, statistical techniques can be applied to define and extract useful information. Professor Moshe Carmeli provides a clear, terse and authoritative introduction to the methods used to do this.

The most important statistical properties are the density of levels and the distributions of their widths, spacings and angular momenta, together with the energy variations of all these quantities. These define the properties of nuclei at high excitations, and also provide the essential data for calculating the cross-sections of many high-energy reactions. Many experimental studies of the statistical properties of nuclei have been made, and these require theoretical explanation.

As Professor Carmeli explains in his introductory chapter, we can understand the statistical properties of nuclei if we renounce any knowledge of their detailed behaviour and see if their properties can be deduced from some very general assumptions. In ordinary statistical mechanics we assume that all the states of a very large ensemble are equally probable, but a different approach must be used to derive the statistics of nuclear levels. Instead of renouncing exact knowledge of the state of the system, we renounce knowledge of the nature of the system itself. A nucleus is considered to be a system of many particles interacting in an unknown way. The statistical mechanics of such ensembles of systems in which all possible laws of interaction are equally probable was first developed by Wigner, and has been extensively applied to analyse nuclear data since then.

If the interaction is known, the energy levels are obtained as the eigenvalues of a matrix. The statistical theory is thus concerned with the properties of states obtained by diagonalizing matrices with elements following postulated distribution laws. The mathematical theory of such distributions, and their implications, forms the core of Professor Carmeli's book. He shows how quite simple distribution functions give states whose statistical properties are in good accord with the experimental data.

The main emphasis is on the mathematics of the connections between statistical theory and random matrices; in addition comparisons are made with experimental data following the recent review of Brody and colleagues. Extensive references to the original literature are provided, together with mathematical appendices on multivariate distributions and the ergodic properties of random matrices. □

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General

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Salary differentials a barrier

In the second of a monthly series of articles, Richard Pearson considers the gulf between salaries in British industry and the universities.*

"PEOPLE transfer may be the best form of technology transfer", as the Advisory Council on Applied Research and Development said in its recent report on the relationship between industry and higher education in the United Kingdom, but very little is known of the extent to which people transfer is hampered by differences, numerical and even structural, between the salaries that people can earn.

Recently I have been looking into recruitment and training policies in microelectronics and biotechnology in Britain. Both technologies are rapidly growing, their products are sold internationally and their job markets are international. Yet there is a marked difference between the reward structures offered by the two technologies, not merely between university and industrial posts but, more surprisingly, between the two new fields of technology.

First, the technology gap. In microelectronics, the entry threshold is a good but not necessarily a first-class first degree. Starting salaries for new graduates in 1983 were about £7,000–£8,500 or even more in some companies. Productivity grows quickly, and earnings of more than £10,000 are possible at the age of 23 and £15,000 or more is possible at 26. One problem is, of course, that such recruits may peak out or hit a career blockage in their mid-thirties but, given the young age profile of the microelectronics industry, few have reached that point yet. But staying up to date is crucial: in one semiconductor company they were saying recently with some alarm that the poor old graduate intake of 1979 was now being left behind by the new intake of 1982!

As it happens, the following week I was discussing the resource needs of the research division of a major pharmaceutical company. "He is only a PhD" said the research director, explaining how recruits are not really worth having until the age of 27 or so, when they will have a PhD and maybe two or three years postdoctoral experience. They might then be paid £10,000 a year, which is at least a significant advance on the salaries of new life science graduates who, if they are lucky, might get a technician job at £6,000 per annum.

This contrast is emphasized by two recent job advertisements. The first was for a section head in the research laboratory (in south-east England) of an international pharmaceutical company. The need was for a PhD, with several years' relevant experience. The expected age was 35-plus. A key role was to innovate and organize the work of others in a section with 16 members of staff, half of them scientists and half technicians. The second was an advertisement by a large multinational semiconductor company for process engineers without line management responsibilities. Here the need was for an electronics degree and two or more years' relevant experience, but managerial experience was not specified.

So who was worth more? The experienced, highly qualified mid-career manager or the relative newcomer? In practice there was little in it. The scientist in the south-east was worth just over £15,000, the engineer, in a low cost-of-living area, around £14,000. Why is it that science requires such a large and personal investment in knowledge before a significant contribution can be made, yet offers the poorer rewards? Salaries, of course, are set by market forces, both employment and product. There are still more people, relative to job openings, queueing up to enter science than electronics. Also, science-based products can take a decade to come to fruition when new electronics widgetry can pay off in a couple of years, witness the massive incomes generated by Apple and Sinclair. In such companies, staff shortages cost big money.

Do these differences matter? Not in general terms, unless you have to run a national pay relativity board or operate in a large unified organization or within a pay structure such as in mainstream higher education. There, life is more constrained. Equity is the watchword, so everyone is paid by reference to a standard structure and in theory neither specialization nor contribution makes much difference. In practice, in high demand subjects, people may be graded at higher points on the scale, if this is possible. Differential speed of promotion is much less easy. Indeed, the chief promotion criterion of publication can be a constraint for commercially orientated innovators and for those whose activities are most closely related to an outside job market.

The safety valve is often supplementary earnings, through consultancy. However, there are often rules restricting consultancy to, say, 20 per cent of a person's time or earnings to 20 per cent of salary. Such requirements may not be unreasonable when seeking to ensure a quantifiable contribution to an institution's teaching and research, but are hardly able to help academics match industrial salaries.

The consequence is that while the top of the lecturer scale, if reached at a relatively young age, may at £14,000 be competitive, entry grades at £7,000–£9,000 can lag significantly behind industrial salaries, especially in the light of the qualifications and experience now demanded for the few vacant academic posts. At the professorial level, earnings become more competitive again, with averages of more than £20,000 per annum.

What we do not know is how this average is made up, and the differences, for example, between subject groups. It is one of the curious anomalies of the British way of life that while most major private sector companies have to publish the numbers of their staff in different earnings bands over £20,000, in the case of our public universities and polytechnics no comparable statistics are available by subject, by institution or even aggregated nationally. The rationale for setting salaries is bound to be complex, specially when research-based activities are involved. But in Britain, very little is known of the outcome. We have chiefly average salaries and scales, advertisements and anecdotes. The extremes and broad patterns may be known or guessed and some crude comparisons can be drawn. But what is clear is that without better information, we cannot tell how imperfect is the British market for research-based jobs and where the barriers to transferability lie. Yet if there is to be more interchange between sectors, especially between higher education and industry, coping with different reward structures is going to be one of the many problems to be overcome. □

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